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(54) Title: *ECK* RECEPTOR LIGANDS

(57) Abstract

Ligands which bind to the *eck* receptor are disclosed. More particularly, polypeptides which bind specifically to the *eck* receptor *eck* receptor binding proteins or EBPs) and DNA sequences encoding said polypeptides are disclosed. Methods of treatment using *eck* receptor ligands and soluble *eck* receptor and disclosed, as are pharmaceutical compositions containing same. A rapid and sensitive method for the detection of receptor binding activity in crude samples is provided.

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ECK RECEPTOR LIGANDS

The invention relates generally to ligands of the *eck* receptor and, in particular, to polypeptide ligands termed *eck* receptor binding proteins (EBPs). Also encompassed by the invention are methods of treatment using *eck* receptor ligands and soluble *eck* receptor, and pharmaceutical compositions containing same. A rapid and sensitive method for the detection of receptor binding activity in crude samples is described.

Background of the Invention

Peptide growth and differentiation factors elicit responses in target cells by means of specific interactions with receptors at the cell surface. Growth factor receptors are typically membrane glycoproteins with distinct extracellular, transmembrane, and intracellular domains. The structural segregation of the domains corresponds to function (Ullrich et al. *Cell* 61, 203 (1990)); the extracellular domain appears to be responsible for ligand binding and ligand-mediated receptor dimerization (Cunningham et al. Science 254, 821 (1991); Lev et al. *J. Biol. Chem.* 267, 10866 (1992)), while the intracellular domain of the receptor, or the intracellular domain of an accessory element (Takeshita et al. *Science* 257, 379 (1992)), is responsible for signal transduction. Much of the specificity of growth factor activity is dictated by the interaction with the binding site on the extracellular domain of the cognate receptor. Chimeric receptors, engineered to contain the extracellular domain of one receptor and the intracellular domain of a second receptor, retain the ligand specificity of the extracellular component (Lehvaslaiho et al. *EMBO J.* 8, 159 (1989)). The downstream signaling pathways

activated by such chimeric receptors correspond to those activated by the intracellular component. In many cases soluble forms of receptors, consisting of only the extracellular domains, retain ligand binding activity (Lev et al. ibid; Duan et al. *J. Biol. Chem.* 266, 413 (1991)). Truncated receptors have been identified in serum (Fernandez-Botran *FASEB J.* 5, 2567 (1991)), cell culture supernatants (Zabrecky et al. *J. Biol. Chem.* 266, 1716 (1991)), and have been produced through recombinant techniques (Lev et al. ibid, Duan et al. ibid).

Recent progress in nucleic acid sequencing and amplification technologies has resulted in the identification of an increasing number of genes which code for previously unidentified growth factor receptors (Wilks *Proc. Natl. Acad. Sci. USA* 36, 1603 (1989); Lai et al. Neuron 6, 691 (1991)). As a result, there is a demand to develop procedures which can define the biological roles of orphan receptors, including techniques which can identify ligands for these receptors (McConnell et al. *Science* 257, 1906 (1992)) Receptor affinity technology is one approach to this problem. This technology may augment existing strategies for the isolation of novel growth factors, since it allows the detection of ligands when biological responses are subtle or undefined.

Recent reports have suggested that the extracellular domains of receptors can be exploited as growth factor-specific affinity reagents. Bailon et al. (*Biotechnology* 5, 1195 (1987)) have shown that the extracellular domain of the IL-2 receptor α subunit can be immobilized on chromatographic media and used for the purification of recombinant IL-2. A genetic fusion of the *kit* extracellular domain with an alkaline phosphatase enzymatic tag allowed the identification of a cell associated ligand for the receptor (Flanagan

et al. *Cell* 63, 185 (1990)). Lupu et al. (*Proc. Natl. Acad. Sci. USA* 89, 2287 (1992).) have reported the affinity purification of an activity which binds to the immobilized extracellular domain of the *erbB-2* gene product.

The *eck* gene, originally identified by cDNA cloning from a human epithelial cell library, encodes a 130 kDa receptor-like protein-tyrosine kinase ($p130^{eck}$) (Lindberg et al. *Mol. Cell. Biol.* 10, 6316 (1990)). Immunohistochemical and mRNA screening of tissues and cell lines suggest that *eck* expression is highest in cells of epithelial origin. By analogy with genes encoding other receptor-like protein-tyrosine kinases, *eck* may be a proto-oncogene and therefore may have a role in carcinogenesis. This potential role for *eck* is more likely given the frequent involvement of epithelial cells in human cancers. Receptor protein kinases are typically activated through interaction with one or more ligands. However, a ligand capable of activating $p130^{eck}$ has not yet been reported. The identification of such a ligand may be important in defining the role of $p130^{eck}$ activation in the development of some human cancers.

It is therefore an object of this invention to identify one or more ligands for $p130^{eck}$. The possible role of $p130^{eck}$ in the transformation of epithelial cells to a cancerous state suggests that identification of the ligand responsible for receptor activation may have therapeutic implications for some epithelial cell-derived malignancies.

SUMMARY OF THE INVENTION

The present invention generally relates to *eck* receptor ligands. More particularly, polypeptides which bind specifically to the *eck* receptor, herein referred

to as *eck* receptor binding proteins (EBPs), are disclosed. EBPs were identified and isolated by affinity chromatography using immobilized extracellular *eck* receptor. EBPs of the present invention may also induce phosphorylation of the receptor upon binding which may trigger changes in target cell physiology, e.g. cell growth and/or differentiation. EBP has an amino acid sequence substantially as shown in SEQ. ID. NO. 1. In a preferred embodiment, EBP has a portion of the amino acid sequence as shown in SEQ. ID. NO. 1. For example, EBP has an amino acid sequence terminating at position 150.

A polypeptide specifically binding the *eck* receptor, wherein the polypeptide has substantially the same amino acid sequence as shown in SEQ. ID. NO. 1 and has a methionine residue at position -1 is also included. By way of example, the polypeptide is [Met^{-1}] EBP^{1-150} or [Met^{-1}] EBP^{1-159}. Also provided for are DNA sequences encoding same. EBPs may also be analogs which have a portion of the amino acid sequence as shown in SEQ. I.D. NO. 1. Examples of such analogs are EBPs terminating at positions 167, 171 or 180.

The invention also provides for EBP as a product of procaryotic or eucaryotic expression of an exogenous DNA sequence, i.e., *eck* receptor binding protein is derived from recombinant DNA methods.

A method for detecting receptor binding activity in crude samples by monitoring the binding to an immobilized ligand binding domain of a receptor is also encompassed by the invention.

Pharmaceutical compositions comprising a therapeutically effective amount of an *eck* receptor ligand are described. Also included is the use of an *eck* receptor ligand in the treatment of certain types of cancers, particularly those characterized by epithelial cell proliferation. *eck* receptor ligands may also be

used for the treatment of wounds to promote healing, for increasing hematopoiesis, and for stimulating the proliferation of hepatocytes and colon crypt cells.

The use of therapeutically effective amounts of ligand antagonists or soluble *eck* receptor for modulating the biological effects of *eck* receptor ligands is also encompassed by the invention. Such treatments are useful in cancer therapy and in the control of inflammation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A. SDS-PAGE analysis of immobilized *eck*-x affinity chromatography. Conditioned medium from HCT-8 cells was concentrated, diafiltered, and loaded onto a 1 ml column of immobilized *eck*-x (1 mg *eck*-x per ml of gel). Samples were concentrated in the presence of 0.02% SDS when necessary; equivalent original volumes are shown in parentheses. Lane 1, Column load (20 ml). Lane 2, Unbound fraction (20 ml). Lane 3, PBS wash (50 ml). Lane 4, pH 4.0 elution, fraction 1 (200 ml). Lane 5, pH 4.0 elution, fraction 2 (200 ml). Lane 6, pH 4.0 elution, fraction 3 (200 ml).

Figure 1B. SDS-PAGE analysis of immobilized *eck*-x affinity chromatography. Cell supernatants from CHO cells transfected with EBP gene were treated and analyzed as described in legend to Figure 1A.

Figure 2. Gel Filtration analysis of purified EBP from HCT-8 cell line. EBP, purified by immobilized *eck*-x receptor affinity chromatography, was amended with 50 µg/ml BSA and injected onto a Superdex 75 column. Fractions were tested for *eck*-x binding activity by BIAcore.

Figure 3. Q-Sepharose chromatography of EBP from CHO cells transfected with EBP cDNA. Samples were analyzed by SDS-PAGE and probed with anti-EBP antibody.

Figure 4. Analysis of release of membrane-bound EBP from CHO cell surfaces by phospholipase C treatment. "-" indicates incubation in the absence of phospholipase C; "+" indicates incubation in the presence of phospholipase C. Time of incubation was 0, 5 and 10 minutes.

Figure 5. Chemical crosslinking of ^{125}I-rEBP to CHO 19.32 cells expressing *eck*. Cells were treated as described in Example 5. Lane 1, ^{125}I-rEBP + CHO 19.32 Lane 2, ^{125}I-rEBP + CHOd- (untransfected). Lane 3, ^{125}I-rEBP + CHO 19.32, no crosslinker added. Lane 4, ^{125}I-rEBP + CHO 19.32 + 50X unlabelled rEBP.

Figure 6. Activation of the *eck* receptor tyrosine kinase in LIM 2405 cells treated with purified rEBP. Serum-starved LIM 2405 cells were treated for 10-15 min at 37°C with increasing concentration of purified rEBP (CHOd-/EBP). The treated cells were lysed and then immunoprecipitated with anti-*eck* C-terminal antibody. The immunoprecipitates were split into two equal samples and resolved in parallel in a 7.5% SDS polyacrylamide gel. The gel was blotted onto membrane, then probed with either monoclonal anti-phosphotyrosine antibody (upper panel) or anti-*eck* C-terminus (lower panel). Migration of the *eck* polypeptide is marked by an arrowhead.

Figure 7. Binding of CHO-derived EBP in various detergent formulations to immobilized *eck* receptor as measured by BIAcore. Purified EBP was diluted to 100 µg/ml in PBS in the presence or absence

of detergent and incubated for 2 hrs. at 3°C. Protein samples were diluted to the concentrations indicated in the figure and tested for *eck* receptor binding.

Figure 8. CFU-C and CFU-Mk formation in bone marrow cultures in the presence of EBP alone and in combination with IL-3, erythropoietin or GM-CSF.

Figure 9. Incorporation of ^{3}H-thymidine in hepatocyte cultures in the presence of EBP, acidic FGF or KGF.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to ligands which bind to the *eck* receptor, a 130 kDa protein tyrosine kinase identified by Lindberg et al., supra. An *eck* receptor ligand may activate the receptor by inducing autophosphorylation by the protein-tyrosine kinase catalytic domain. Protein-tyrosine kinases are part of the signal transduction pathway which modulates cell proliferation and differentiation. Therefore, a ligand capable of activating protein-tyrosine kinase activity of the *eck* receptor will likely be important in modulating the growth and differentiation of cells expressing the receptor. An *eck* receptor ligand may be a polypeptide, peptide, or non-protein molecule which binds to and/or activates the *eck* receptor.

The present invention provides for novel polypeptides which specifically bind to the *eck* receptor. These polypeptides are referred to as *eck* binding proteins, or EBPs. EBPs have been isolated from the conditioned medium of SK-BR-3 and HCT-8 cell lines by receptor affinity chromatography using the *eck* receptor extracellular domain (*eck*-x) as the affinity reagent. Construction of the *eck*-x gene and expression

and purification of *eck*-x are described in Example 1. The purification of *eck* binding proteins on immobilized *eck*-x is described in Example 3A.

EBP derived from the HCT-8 cell line exists in several different molecular weight forms in the range of 21-27 kDa as revealed by SDS-PAGE (Figure 1A). N-terminal sequencing of EBPs in the 21-27 kDa range revealed a single sequence (Example 3B). After enzymatic removal of carbohydrate chains, a mixture of species in the range of 17-19 kDa was observed, suggesting that the different forms may result from alternative post-translational processing of the protein.

The N-terminal sequence of SK-BR-3 derived EBP was identical to the N-terminal amino acid sequence predicted from the expression of the B61 gene (Holzman et al. *Mol Cell. Biol.* 10, 5830 (1990); PCT Application No. WO 92/07094). The B61 gene was originally identified by differential hybridization as an immediate-early response gene and its expression was induced in cultured human vascular endothelial cells by treatment with tumor necrosis factor-α. Based upon the sequence of the EBP gene, the encoded protein was predicted to have 187 amino acids in its mature form. The isolation and characterization of the EBP-encoded protein has not been previously reported. It was proposed that the EBP protein functions as a cytokine-induced marker for inflammation and therefore is useful as a diagnostic reagent for the detection of an impending inflammatory response (PCT Application No. WO 92/07094).

cDNA encoding EBP having the N-terminal sequence determined in Example 3B was cloned and sequenced and, as expected, found to be identical to the B61 gene (Holzman et al. supra). The B61 DNA sequence reported by Holzman et al. is shown in SEQ. ID. NO. 11.

The EBP (or EBP gene) was expressed in CHO cells and in *E. coli* as described in Example 4. At least two polypeptides having different molecular weights were expressed by the EBP gene in CHO cells. C-terminal sequencing of CHO cell EBP revealed only the sequence -lys-arg-leu-ala-ala-COOH which indicated a polypeptide of 150 amino acid residues. This polypeptide is referred to as EBP^{1-150}. A polypeptide of 187 amino acids corresponding to the predicted EBP protein, if present at all, represents a very minor product of CHO cell expression. Expression of the EBP gene (lacking the leader sequence) in *E. coli* resulted in a single product on SDS-PAGE having a C-terminal sequence predicted for the full-length protein. This polypeptide which has a methionine residue at the amino terminus is designated $[met^{-1}]$ EBP^{1-187} and, with the exception of the N-terminal met, is identical in amino acid sequence to the predicted EBP protein.

CHO cell-derived rEBP has been shown to interact with the *eck* receptor by the following experiments (see also Example 5): 1) Crosslinking of CHO rEBP to colon carcinoma cells naturally expressing the *eck* receptor or to CHO cells transfected with the *eck* gene; 2) equilibrium binding studies of CHO rEBP to *eck* receptors on colon carcinoma cells; and 3) stimulation of *eck* receptor phosphorylation on colon carcinoma cells. Induction of receptor phosphorylation by CHO rEBP indicates that the ligand may be able to effect a biological response (e.g., growth or differentiation) in cells displaying the *eck* receptor.

It is apparent that EBP may be expressed in a number of different molecular weight forms, more than one of which may be biologically active. Various forms of EBP are produced naturally by human cell lines and by EBP gene-transfected host cells as shown in Figures 1A and 1B. As shown in Figure 3, EBP from transfected CHO

cells was isolated as two different molecular weight forms of 22 kDa (major) and 24 kDa (minor). Characterization of these different forms revealed two distinct EBPs of 150 and 165 amino acids, designated EBP^{1-159} and EBP^{1-159}. Phosphoinositol-phospholipase C treatment of EBP-transfected CHO cell lines releases soluble EBP, strongly suggesting a glycolipid form of EBP. Isolated 27 kDa forms of EBP are susceptible to digestion with phospholipase D further suggesting that these forms are solubilized forms of glycophospholipid-anchored EBP.

The invention provides for EBP having the activity of specifically binding to the *eck* receptor and having substantially the same amino acid sequence as shown in SEQ. ID. NO. 1. The term "substantially the same amino acid sequence" as used herein refers to deletions or substitutions of amino acids in SEQ. ID. NO. 1 such that the resulting polypeptides specifically bind the *eck* receptor. As described above, EBP also induces phosphorylation of the *eck* receptor. However, the present invention encompasses polypeptides which bind the *eck* receptor and may or may not induce receptor phosphorylation. In a preferred embodiment, EBP has a portion of the amino acid sequence as shown in SEQ. ID. NO. 1. For example, EBP has the amino acid sequence as shown in SEQ ID. NO. 1 terminating at position 150 or has substantially the same amino acid sequence as shown in SEQ. ID. NO. 1 Preferably, EBP is EBP^{1-150}, that is, it has the amino acid sequence from positions +1 to 150 as shown in SEQ. ID. NO. 1.

An EBP which has substantially the same amino acid sequence as shown in SEQ. ID. NO. 1 and has a methionine residue at position -1 is also included. $[Met^{-1}]$ EBP^{1-150} and $[Met^{-1}]$ EBP^{1-159} are examples. Also provided for are DNA sequence encoding same. A truncated DNA sequence encoding amino acid residues +1

to 150 as shown in SEQ. ID. NO. 1 was constructed and expressed in *E. coli*. The resulting protein, [met^{-1}] EBP$^{1-150}$, binds to the *eck* receptor and induces receptor phosphorylation (Example 6).

Also encompassed by the invention are fragments and analogs of the polypeptide encoded by the amino acid sequence shown in SEQ. ID. NO. 1 wherein said fragments and analogs bind to the *eck* receptor, and DNA sequences encoding said fragments and analogs. Included are fragments having deletions from the N-terminal or C-terminal ends of the polypeptide as shown in SEQ. ID. NO. 1 and deletions from internal regions. Examples of EBP fragments include EBP$^{1-167}$, EBP$^{1-171}$ and EBP$^{1-180}$ described in Example 5. Analogs include amino acid substitutions at one or more sites in the polypeptide. Fragments and analogs of the invention are readily constructed using recombinant DNA techniques which are known to those skilled in the art. The biological activity of the resulting fragments and analogs is readily tested by binding to *eck* soluble receptor or to *eck* receptors on cell surfaces and by inducing phosphorylation of the *eck* receptor.

The invention also includes EBP characterized by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence, i.e., EBP is recombinant EBP. Exogenous DNA sequences may be cDNA, genomic or synthetic DNA sequences. EBP may be expressed in bacterial, yeast, plant, insect or mammalian cells in culture or in transgenic animals. DNA vectors suitable for the expression of EBP in a variety of host cells are known to one skilled in the art. Examples of such vectors are pDSRα2 for the expression of EBP gene in CHO D- cells and pCFM1156 for the expression of EBP gene in *E. coli*.

EBP expression in *E. coli* results in the formation of insoluble inclusion bodies. Recovery of

biologically active EBP requires solubilization of EBP aggregates followed by refolding of the solubilized protein. Example 4C describes a refolding procedure for *E. coli* derived EBP which yields EBP active in *eck-x* binding and *eck* phophorylation. EBP refolding procedures may be modified to increase the activity of the renatured protein and to increase the yield of active EBP. Such modifications include changing the denaturant used to solubilize the inclusion body (e.g. using guanidinium chloride vs. urea), the oxidizing agent (which may include oxidation reduction pairs such as oxidized and reduced glutathione), or the dilution protocol from the denaturant (e.g. EBP may be diluted at a different protein concentration into a detergent containing buffer at a modified pH).

Also provided by the invention is a method for detecting a ligand present in crude samples, e.g., conditioned medium, which is capable of binding a receptor. The method comprises the steps of:

a) immobilizing a purified ligand binding domain of the receptor;

b) contacting the immobilized receptor with conditioned medium containing the ligand; and

c) monitoring the binding of the ligand to the immobilized receptor by a surface plasmon resonance detection system.

As described in Example 2, this method provides a rapid and sensitive screening for *eck* receptor binding activity in cell supernatants. The results of this screening are shown in Table 1. Although the method is used to detect *eck* binding activity, it may be generally applied to any receptor-ligand interaction. Any ligand binding domain of a receptor may be immobilized for the isolation of receptor binding proteins. In a preferred embodiment, the ligand binding domain is the extracellular domain or

a fragment or analog thereof which is competent for binding. In addition to screening cell supernatants, the method may also be used to screen mixtures of random sequence peptides for receptor binding.

The invention provides, for the first time, a method of modulating the endogenous enzymatic activity of an *eck* receptor. Said method comprises administering to a mammal an effective amount of a ligand to the *eck* receptor to modulate the enzymatic activity of said receptor. *eck* receptor enzymatic activity regulates cellular functions comprising differentiation, proliferation and metabolism. In a preferred embodiment, EBP, or a fragment or analog thereof, is the ligand. However, other ligands may also be used in modulating *eck* receptor activity, for example, polypeptides not related to EBP, or peptides and non-protein organic molecules.

Also encompassed by the invention is a method for identifying compounds that modulate the activity of an eck receptor. Said method comprises the steps of:

a) exposing cells exhibiting the receptor to known ligands for a time sufficient to allow formation of receptor-ligand complexes and induce signal transduction;

b) determining the extent of activity within the cells; and

c) comparing the measured activity to the activity in cells not exposed to the ligand.

Eck receptor activity may be detected by changes in target cell proliferation, differentiation or metabolism. A description of methods relating to the modulation of *eck* receptor activity on hematopoietic progenitor cells, colon crypt cells and hepatocytes appears in Example 9.

The invention also encompasses an isolated *eck* receptor-ligand complex which results from the

interaction of the *eck* receptor with a ligand such as EBP. The interaction may result in activation of the receptor and transduction of a signal which modulates the physiological state of the receptor-bearing cells. Preferably, the ligand acts as a growth factor to stimulate the proliferation of target cells. Alternatively, ligand binding may not activate the eck receptor. In this instance, the ligand may act as an antagonist for other molecules which activate the receptor and induce in signal transduction.

The *eck* receptor is expressed primarily in tissues containing significant amounts of epithelial cells (e.g. lung and intestine) and in cell lines derived from epithelial cells (Lindberg et al., supra). A ligand of the *eck* receptor may stimulate either the growth or differentiation of cells expressing the receptor. A ligand which induces differentiation of cells bearing the *eck* receptor may be useful in the treatment of certain types of cancers, particularly those resulting from proliferation of epithelial cells. An *eck* receptor ligand may be used alone or in combination with standard chemotherapy or radiation therapy for cancer treatment.

EBP interaction with the *eck* receptor may be involved in the development of a cancerous state through stimulation of cell growth or may promote metastasis by stimulating cell mobility and adhesion. Several strategies are available for modulating the biological effects of EBP. Fragments or analogs of EBP which bind to but do not activate the *eck* receptor are useful as EBP antagonists. Administration of an EBP antagonist having affinity for the *eck* receptor will block receptor binding and activation by circulating EBP. Administration of soluble *eck* receptor may also be used to counteract the biological effects of EBP. Soluble *eck* receptor will compete with cell surface receptors

for binding to EBP and thereby reduce the extent of *eck* receptor activation by EBP. Soluble *eck* receptors suitable for therapeutic use include the receptor protein described in Example 1 as well as fragments and analogs thereof which bind EBP. In addition, monoclonal antibodies directed either to EBP or to the *eck* receptor may be useful in blocking the interactions of EBP with *eck* receptors on cell surfaces.

Expression of the EBP gene in endothelial cells has been shown to be stimulated by TNF-α and IL-1β, two proinflammatory cytokines which activate various functions in endothelial cells as part of the inflammatory response (Holzman et al. <u>supra</u>). A treatment comprising the administration of soluble *eck* receptor to reduce levels of EBP that are increased during the inflammatory response may be useful in controlling inflammation.

A method for the treatment of a wound in a mammal comprising administering a therapeutically effective amount of an *eck* receptor ligand is provided. As shown in Example 9A, EBP promoted an increase in tissue wet weight, total protein, total DNA, and total glycosaminoglycan in the rat wound chamber assay. Since EBP is expressed early in the inflammatory response, it could play a role in the recruitment of epithelial cells to the site of an injury.

A method for increasing hematopoiesis in a mammal comprising administering a therapeutically effective amount of an *eck* receptor ligand is also provided. As shown in Example 9B, EBP in combination with interleukin-3 (IL-3) shows a significant enhancement of CFU-Cs in mouse bone marrow cultures. EBP would be useful in restoring hematopoiesis when myelosuppression has occurred, either as a result of a disease or after exposure to myelosuppressive agents, such as chemotherapeutic drugs or agents. In a

preferred embodiment, a therapeutically effective amount of EBP is used in combination with a therapeutically effective amount of IL-3 for increasing hematopoiesis.

Also included is a method for stimulating the proliferation of colon cells in a mammal comprising administering a therapeutically effective amount of an *eck* receptor ligand. As shown in Example 9C, EBP stimulates cell proliferation in a colon crypt assay. An eck receptor ligand such as EBP would be useful in alleviating gut toxicity following chemotherapy.

A method for stimulating proliferation of hepatocytes comprising administering a therapeutically effective amount of an *eck* receptor ligand is provided. Example 9D shows stimulation of hepatocytes by EBP. This stimulation is comparable to that seen in the same assay with acidic fibroblast growth factor (aFGF), a known hepatocyte growth factor. Treatment with an eck receptor ligand is useful for repairing liver damage resulting from disease or injury.

The invention provides for pharmaceutical compositions comprising therapeutically effective amounts of an *eck* receptor ligand together with pharmaceutically acceptable diluents, carriers, preservatives, emulsifiers and/or solubilizers. A "therapeutically effective amount" as used herein refers to that amount which provides therapeutic effect for a given condition and administration regimen. It is expected that one skilled in the art would be able to determine a therapeutically effective amount of an *eck* receptor ligand for any given condition being treated.

Pharmaceutical compositions include diluents of various buffers (e.g., Tris, acetate, phosphate), solubilizers (e.g., Tween, Polysorbate), carriers such as human serum albumin, preservatives (thimerosol, benzyl alcohol) and anti-oxidants such as ascorbic acid. As shown in Example 7, the ability of purified and

diluted EBP to bind to soluble *eck* receptor is prolonged when EBP is formulated in the presence of a stabilizing agent. The stabilizing agent may be a detergent, such as tween-20, tween-80, NP-40 or Triton X-100. EBP may also be incorporated into particulate preparations of polymeric compounds for controlled delivery to a patient over an extended period of time. A more extensive survey of components in pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1990).

EBP may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral or nasal administration. The route of administration will depend upon the particular condition being treated.

The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

EXAMPLE 1

Production of *eck* receptor extracellular domain

A. Construction of an *eck*-x expression plasmid

A plasmid for mammalian expression of the extracellular domain of *eck* was generated in several steps beginning with pGEM3Z-*eck*, a 3.4kb EcoRI-Kpn I subclone of the *eck* cDNA (Lindberg et al. <u>supra</u>). Oligonucleotides 317-11 (5'-AGCTTAGATCTCC-3'; SEQ. ID. NO. 7) and 317-12 (5'-AATTGGAGATCTA-3'; SEQ. ID. NO. 8) were kinased and ligated to the 1.7kb EcoRI fragment of pGEM3Z-*eck* and pGEM4Z which had been digested with Hind III and EcoRI. A clone was selected which had the oligonucleotides added only to the 5' end of *eck*. Characteristics of this clone include the addition of Hind III and Bgl II sites and deletion of the 5' EcoRI

site adjacent to the *eck* sequence. The insert from this clone was isolated following digestion with Hind III and EcoRI and ligated to kinased oligonucleotides:

317-9 5'-AATTCCAGACGCTGTCCCCGGAGGGATCCGGCAACTGAG-3' (SEQ. ID. NO. 9) and

317-10 5'-TCGACTCAGTTGCCGGATCCCTCCGGGGACAGCGTCTGG-3 (SEQ. ID. NO. 10)

with pGEM4Z digested with Hind III and Sal I. This added a Bam H1 site, a TGA stop codon following Asn^{534} and a Sal I restriction enzyme site. The Hind III-Sal I fragment containing the coding sequence for the external domain of *eck* was then transferred to pDSRα2 (deClerk et al. *J. Biol. Chem.* <u>266</u>, 3893 (1991)).

B. Mammalian cell expression of *eck* x

The expression plasmid pDSRα-*eck*-x was introduced in CHO cells by calcium mediated transfection (Wigler et al. *Cell* <u>11</u>, 233 (1977)). Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid and one clone, designated 21.021 was chosen for amplification. Expression of the *eck* gene was monitored by RNA hybridization (Hunt et al. *Exp. Hematol.* <u>19</u>, 779 (1991)).

Amplification of *eck* expression was done in 10 nM methotrexate. Cell line 21.02 was expanded for production of *eck*-x. Roller bottles were seeded at 2 x 10^7 cells in 200 ml DMEM:Ham's F12 (1:1) supplemented with non-essential amino acids, 10 nM methotrexate and 5% FBS. Cells reached confluence in 3 days at which time fresh media lacking 5% FBS was added. Conditioned media was harvested and replaced after seven days and a second harvest was taken after fourteen days.

C. Purification of *eck*-x

Conditioned medium from 21.02 cells was concentrated and diafiltered against 10 mM Tris-HCl, pH 7.4 using a 10,000 MWCO spiral wound filter (S1Y10, Amicon, Danvers, MA). The 50 mL concentrate was loaded onto an anion exchange column (Hema-Q, 10 µm particle size, 1.6 x 12 cm, Separon, Sunnyvale, CA) and eluted with a linear gradient of 0-0.5 M NaCl in 10 mM Tris-HCL, pH 7.4. Fractions were analyzed by SDS-PAGE and western blotting using a rabbit antiserum generated against a synthetic N-terminal peptide of *eck*-x. Fractions containing *eck*-x were pooled, dialyzed against 10 mM Tris-HCl, pH 7.4, reloaded onto the Hema-Q column, and eluted and analyzed as before. The resulting pool was concentrated to 3 mL (centriprep-10, Amicon, Danvers, MA) and applied to a Superdex 200 (Pharmacia, Piscataway, NJ) gel filtration column (2.2 x 90 cm, flow rate 1.0 ml/min) equilibrated in PBS. A pool containing the purified *eck*-x was made and served as the basis of further experiments.

EXAMPLE 2

Screening of conditioned media for binding to the *eck* extracellular domain

Interactions with *eck*-x were measured on a surface plasmon resonance detector system (BIAcore, Pharmacia Biosensor, Piscataway, NJ) using procedures recommended by the manufacturer. The dextran surface of the sensor chip was activated by injecting 35 µl of 0.2M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl, 0.05M N-hydroxysuccinimide at a flow rate of 5 µl/min. The purified *eck*-x (0.2 mg/ml in 10 mM sodium acetate, pH 4.0) was immobilized by two consecutive 50 µl injections at 5 µl/min. Unreacted binding sites were blocked by injection of 1M ethanolamine, pH 8.5. The

surface was washed overnight in running buffer (HBS, 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.4) until a stable baseline was achieved. Typical immobilizations resulted in the establishment of baselines 6000-8000 response units above original values. 50 µl samples of various conditioned media, cultivated under serum free conditions and concentrated five- to forty-fold (centricon-10, Amicon, Danvers, MA), were injected at a flow rate of 10 µl/min. Sample response was measured at report points on the sensorgram corresponding to 20 seconds after the conclusion of each injection. The immobilized *eck*-x surface was regenerated between samples by 50 µl injections of 25mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 10.4.

Purified *eck*-x immobilized on a BIAcore sensor chip was used to screen concentrated conditioned media for receptor binding activity. Binding activity was observed in several conditioned media, including those from HCT-8, SK-BR-3, and HT-29 cell lines. (see Table 1)

Table 1. Testing of cell culture supernatants by BIAcore and anti-EBP Western blot

Cell line	Concentration	*eck*-x Binding Activity (BIAcore Response units)	Anti-EBP Western
A704	40X	49	-
TE671	40X	115	-
CCD18CO	40X	25	-
CCFSTTG1	40X	35	-
HCT-8	40X	180	++
GMO-0948	40X	10	-
SK-BR-3	40X	270	++
HT-29	40X	310	++
MDA-MB-453	30X	80	++
FF-1	30X	55	-
WS-1	25X	55	-
BRL-3A	25X	125	-
GMO1391	25X	20	-
HT-1080	20X	230	+
AG-2804	20X	170	+
BUD-8	20X	-10	-
AG-3022	20X	175	+
HFL-1	20X	15	-
LIM-1863	20X	75	-
PAEC	20X	-10	-
HOs	10X	95	-
33C0	10X	-10	-

Samples (50 µl injections) were tested for binding to immobilized *eck*-x using BIAcore, as described in Example 2. Aliquots (10 µl) of the same samples were analyzed retrospectively with rabbit anti-EBP (*E. coli*) antiserum.

EXAMPLE 3

Purification and Characterization of an *eck* receptor binding protein (EBP) from conditioned medium.

A. Immobilized *eck*-x receptor affinity chromatography.

Purified *eck*-x was dialyzed against 0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3 and brought to a final concentration of 2 mg/ml. The protein was immobilized on CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) at a ligand density of 1 mg *eck*-x per ml of gel (Kenny et al. in *New Protein Techniques*, J.M. Walker, ed. The Humana Press, Clifton, NJ. (1988)).

Conditioned medium from SK-BR-3 or HCT-8 cell lines was concentrated twenty fold and diafiltered against PBS (S1Y10 spiral cartridge, Amicon). The concentrate was loaded directly onto columns of immobilized *eck*-x (1 x 1 cm, 1mg *eck*-x per ml resin) at a flow rate of 0.1 ml/min. The column was washed with 10 ml of PBS, followed by elution with 0.05 M sodium acetate, 0.5 M NaCl, pH 4.0. The elution pool was brought to 0.01% SDS, concentrated, and buffer exchanged against 10 mM Tris-HCl, pH 8.0 in a centricon-10 ultrafiltration device (Amicon). Samples were mixed with SDS-PAGE sample buffer, harvested from the centricon-10, and loaded directly onto polyacrylamide gels. Gels were stained with silver (Merrill *Meth. Enzymol.* 182, 477 (1991)) or blotted onto PVDF membranes (Problot, Applied Biosystems, Foster City, CA) for N-terminal sequence analysis (Fausset et al. *Electrophoresis* 12, 22 (1991)).

An SDS-PAGE analysis of a typical run is shown in Figure 1. The pH 4.0 elution of the column, shown in lane 5, displays a significant enrichment for several proteins with apparent molecular weights of 21-27 kDa.

These proteins were not detected when a similar volume of unconditioned medium was passed over the same column, indicating that the 21-27 kDa proteins were produced by the cell lines. In contrast, the higher molecular weight proteins present in the pH 4.0 elution fractions of HCT-8 or SKBR-3 conditioned medium were also observed in the unconditioned medium. These proteins represent nonspecific interactions with the column, and originate from the serum used for cell culture.

B. N-terminal sequence analysis of EBP.

N-terminal sequence analysis was performed on a liquid-pulse automatic sequencer (model 477, Applied Biosystems, Foster City, CA). The resulting phenylthiohydantoinyl amino acids were analyzed by on-line microbore high performance liquid chromatography (Model 123, Applied Biosystems) using a Brownlee C-18 reverse-phase column (0.21 x 25 cm). The sequencing cycles and optimization for sequence analysis of PVDF blots were based on recommendations supplied by Applied Biosystems.

N-terminal sequence analysis of the electroblotted proteins in the 21-27 kDa region of the gel revealed a single sequence (SEQ. ID. NO. 13):

NH_2-Asp-Arg-His-Thr-Val-Phe-Trp-[Asn]-Ser-Ser-Asn-Pro-Lys-Phe-Arg-Asn-Glu-Asp-Tyr-Thr-Ile-His-Val-Gln

A computer based homology search of the NBRF protein database (Devereux et al. *Nucleic Acids Res.* 12, 387 (1984)) resulted in the unambiguous assignment of this sequence to EBP (Holzman et al. supra).

C. Structural characterization of EBP.

Since N-terminal sequencing detected a single sequence, the multiple forms of EBP observed by SDS-PAGE

probably arise from post-translational modifications at other regions of the molecule. The sequencing yield of cycle 8 (N) was greatly diminished, indicating efficient glycosylation at this site. However, the apparent heterogeneity of the protein may not be fully attributable to glycosylation differences, since digestion of rEBP with combinations of N-glycanase, neuraminidase, and O-glycanase resulted in a mixture of forms with M_r 17-19 kDa, when analyzed by SDS-PAGE. Since the EBP gene codes for a protein of 22kDa (Holzman et al. ibid), this observation suggested that EBP might be subject to proteolytic processing.

D. Interactions of EBP with soluble *eck* receptor.

Gel filtration analysis of the pH 4.0 eluted pool demonstrated that all of the *eck*-binding activity, as measured by BIAcore response, could be attributed to material eluting with apparent molecular weight of 22 kDa (Figure 2). SDS-PAGE analysis of the fractions from this column confirmed that EBP was co-eluted with the receptor binding activity. In separate experiments, purified EBP did not bind to BIAcore surfaces activated with the extracellular domain of the kit receptor, although these surfaces could bind rSCF, the kit ligand.

E. Screening of additional cell lines for *eck* binding proteins.

Following the isolation and identification of EBP, antiserum to the protein was prepared, and a retrospective analysis of the original screening was performed (Table 1). Western blot analysis confirmed that EBP was present at high levels in three conditioned media (SK-BR-3, HCT-8, and HT-29) which scored positive in the screening. Several other cell lines (AG-3022,

AG-2804, and HT-1080) scored positive, but only trace amounts of processed EBP could be detected by the Western analysis. These cell lines did produce a 28kDa protein which was detected by the anti-EBP antiserum.

EXAMPLE 4

Cloning, Expression and Characterization of EBP

Heat-disrupted phage from a human umbilical vein endothelial cell (HUVEC) library (Clontech Laboratories, Palo Alto, CA) were used as a template for amplification of the human EBP gene by polymerase chain reaction (PCR) (Saiki et al. *Science* 230, 1350 (1985); Mullis et al. Cold Spring Harbor *Symp. Quant. Biol.* 51, 263). Primers were designed based on the published nucleic acid sequence of EBP (Holzman et al. ibid) to yield PCR fragments that could be inserted into either *E. coli* or CHO cell expression vectors.

A. Cloning of EBP for *E. coli* Expression

A gene for *E. coli* expression of the full length form of EBP was generated by PCR using oligonucleotide primers 386-4 and 386-5 as shown below:

386-4) 5' AAG CAT ATG GAT CGC CAC ACC GTC TTC TGG 3' (SEQ. ID. NO. 2)

386-5) 5' GAA GGA TCC TTA TCA CGG GGT TTG CAG CAG CAG AA 3' (SEQ. ID. NO. 3)

This form lacked the signal peptide and included an initiator methionine as well as restriction sites necessary for cloning into the expression plasmid pCFM1156 (Fox et al. *J. Biol. Chem.* 263, 18452 (1988)). A 10 µl aliquot of the λ gt11/HUVEC library (Clontech) was heat treated at 70° C for 5 minutes then quick-cooled on wet ice. The disrupted phage were used as the

template for a PCR reaction containing 300 picomoles each of primers 386-4 and 386-5, 1X TaqI polymerase buffer (Promega, Madison, WI), 0.77 mM of each dNTP, and 2.5 units of TaqI polymerase (Promega) in a volume of 100 ul. The product of this reaction was extracted with phenol/chloroform, ethanol precipitated, resuspended in 20 ul of distilled water and then digested with the restriction endonucleases NdeI and BamHI (Boehringer Mannheim, Indianapolis, IN). This fragment was ligated into the plasmid vector pCFM1156 which had been digested with the same two enzymes and transformed into the *E. coli* host strain FM5 (A.T.C.C. No. 53911). When transformed cells were temperature shifted from 30°C to 42°C, EBP was expressed at high levels.

A gene designed to express the 150 amino acid form of EBP in *E. coli* was constructed as described for the full-length gene except that oligonucleotide 469-11 was used in PCR instead of 386-5. Oligonucleotide 469-11 has the sequence:

5′ GAAGGATCCCTATTATGCTGCAAGTCTCTTCTCCTG 3′
(SEQ. ID. NO. 4)

B. Cloning of EBP for CHO cell expression

Total RNA was isolated from the cell line SK-BR-3, and used to prepare cDNA. Three µg of total RNA was mixed with 3 µg of random primer (Gibco BRL, Gaithersburg, MD), incubated at 65°C for 5 min, then cooled briefly on ice. The RNA-primer mixture was then used in a cDNA reaction which consisted of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 125 µM of each dNTP (dATP, dCTP, dGTP, dTTP), 200 units of reverse transcriptase (Superscript, BRL), in a final reaction volume of 20 µl. The reaction was incubated at 37°C for 1 h.

Two oligonucleotides were synthesized and used with the SK-BR-3 cDNA to amplify the EBP coding region by PCR.

386-2) 5' GAA TTC AAG CTT CAG GCC CCG CGC TAT GGA G 3' (SEQ. ID. NO. 5)

386-3) 5' GAA TTC TCT AGA TCA TCA CGG GGT TTG CAG CAG CA 3' (SEQ. ID. NO. 6)

The PCR contained 1 µl of the cDNA reaction, 500 ng of both of the above oligonucleotides, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM of each dNTP, and 1.25 units of Taq polymerase (Perkin Elmer Cetus, CA). DNA was amplified for 35 cycles (94°C for 30 s, 50°C for 1 min, 72°C for 1 min), extracted 1 time with phenol, 1 time with phenol-chloroform, precipitated, pelleted by microcentrifugation, and digested with the restriction enzymes Hind III and Xba I (Boerhinger Mannheim). The DNA was gel-purified (Geneclean II, Bio 101, La Jolla, CA) and ligated to the plasmid pDSRα2 (deClerck et al., ibid) which had been previously digested with the same restriction enzymes. The ligated DNA was transfected into competent HB101 bacteria (BRL), and plasmid DNA was isolated (Qiagen, Chatsworth, CA). The DNA sequence was confirmed by the dideoxy chain termination reaction (Sanger et al. *Proc. Natl. Acad. Sci. USA* 74 5463 (1977)) on double-stranded plasmid DNA using synthetic primers that corresponded to the EBP DNA sequence.

Culture supernatants from CHO cells transfected with the EBP gene displayed *eck*-x binding activity on the BIAcore, and EBP could be recovered from the supernatants by immobilized *eck*-x receptor affinity chromatography. Untransfected CHO cells, or CHO cells transfected with a EBP gene containing an internal deletion, displayed no receptor binding activity.

C. Purification of recombinant EBP from *E. coli* and CHO cells

Recombinant EBP was purified from CHO cell culture supernatants by immobilized *eck*-x receptor affinity chromatography as described in Example 3A. The purified EBP was dialyzed vs. PBS with either 5 mM CHAPS (for structural analysis and crosslinking studies) or 0.5 mg/ml BSA (for phosphorylation studies). The CHO cell-derived EBP purified by receptor affinity chromatography contained two major bands, as well as a few minor bands (Figure 1B).

Recombinant EBP from CHO cells was also purified by conventional chromatography. The CHO cell culture supernatant was concentrated and diafiltered against 10 mM Tris, pH 8.5 and applied to an anion exchange column (Q-Sepharose, Pharmacia-LKB). The column was eluted with a linear gradient of NaCl in 10 mM Tris-HCl, pH 8.5. Analysis of the fractions by western blotting showed that the two major EBP bands had been separated from one another. Separate pools were made of fractions containing the major EBP bands, and the pools were further purified by gel filtration chromatography (Superdex-75, Pharmacia-LKB)

Recombinant EBP from *E. coli* was purified by the following method. Cells expressing the EBP^{1-150} or EBP^{1-187} genes were suspended in 10 mM Tris-HCL, pH 7.4 and lysed using a French press. The lysate was centrifuged in a J6-B (JS-4.2 rotor) at 4000 rpm for 30 min. The insoluble pellets (containing either unfolded EBP^{1-150} or EBP^{1-187}) were saved for further processing. The pellets were suspended in 2% sodium deoxycholate, 5 mM EDTA, 10 mM Tris-HCl, pH 8.5, and mixed for 30 minutes at 4°C. The suspensions were centrifuged as above, and the supernatants discarded. The insoluble pellets were suspended in 10 mM Tris-HCl, pH 7.4, mixed, and centrifuged as above. The insoluble

pellets were dissolved in 2% sarkosyl, 10 mM CAPS, pH 10 in order to solubilize EBP^{1-150} or EBP^{1-187}. $CuSO_4$ was added to a final concentration of 50 μM, and the mixtures were stirred overnight at 4°C, then treated with Dowex 1X4 resin in order to remove the detergent.

SDS-PAGE analysis revealed that a large proportion of EBP^{1-150} or EBP^{1-187} had oxidized and was monomeric. However, gel filtration analysis of EBP^{1-187} showed that the protein behaves as a high molecular weight noncovalent aggregate. In contrast, gel filtration analysis indicates that the refolded EBP^{1-150} behaves as a monomer or dimer.

EBP^{1-187} and EBP^{1-150} produced in *E. coli* were tested for binding to immobilized *eck*-x by BIAcore as described in Example 2. The EBP^{1-187} aggregate bound poorly, if at all, to the *eck*-x surface. Refolded EBP^{1-150} demonstrated high affinity for *eck*-x surfaces on the BIAcore.

Alternatively, the following procedure was used to re-fold EBP^{1-150} expressed in E. coli. Cell paste was suspended in 9 volumes (v/w) of cold Super-Q water. The suspended cell paste was lysed using a Gaulin homogenizer at a pressure of 9,000 psi. The lysate was immediately centrifuged at 3,500 X G, 4°C for 30 minutes. The supernatant was discarded and the pellet, containing EBP inclusion body, was saved. The pellet was suspended in 10 volumes (v/w) of 8M urea, 0.1M Tris, pH 8.5, and stirred for one hour. Centrifugation was then performed to remove the insoluble fraction. Refolding was effected by two stepwise dilutions of the soluble inclusion body. First, the inclusion body was diluted into 10 volumes (v/w) of 3M urea, 0.1M Tris, pH 8.5, containing 0.0005% $CuSO_4$ as a oxidizing agent, at 4°C while stirring overnight. This material was diluted with 3 volumes (v/v) of 20mM Tris, pH 9.2, and was incubated for 24 hrs with gentle stirring.

Centrifugation was performed at 15,000 X G, 4°C for 30 minutes to remove precipitate.

The supernatant was then applied into Q-Sepharose Fast Flow column and washed with five column volumes of 20mM Tris, pH 9.2. The column was eluted with a linear gradient of NaCl from 0-0.5M in 20mM Tris, pH 9.2. Fractions containing EBP were pooled, concentrated, and subjected to Superdex-75 chromatography. The EBP was eluted with 1X PBS.

The resulting purified EBP had a specific activity that is 30-40% of purified CHO derived EBP as measured by its ability to bind to immobilized *eck*-x in a BIAcore assay. The *E.coli* produced EBP refolded and purified by this procedure induced the phosphorylation of *eck* localized on cellular surfaces.

D. Characterization of recombinant EBP from *E. coli* and CHO cell expression

Recombinant EBP, purified by receptor chromatography from CHO cell culture supernatants, or by RP-HPLC from *E. coli* was digested with trypsin and analyzed by RP-HPLC. Although the C-terminal peptide (aa 155-187) was recoverable from the EBP^{1-187} gene expressed in *E. coli*, it could not be detected in the mammalian derived recombinant protein. Carboxypeptidase digestion of the purified CHO EBP indicated that the only detectable C-terminal sequence was -lys-arg-leu-ala-ala-COOH (SEQ. ID. NO. 12), indicating a terminus at amino acid 150.

E. Alternative Forms of EBP

EBP isolated from SK-BR-3 cell line as described in Example 3 migrated on SDS-PAGE in the range of 21-27 kDa (see Figure 1A). Recombinant EBP in CHO cell supernatants existed as two major species and a few minor bands after *eck*-x chromatography (Example 4 and

Figure 1B). Further characterization of the different molecular weight forms of CHO-derived EBP was undertaken. Purification of recombinant EBP from CHO cell supernatants revealed two bands of 22 and 24 kDa and a third minor band of 27 kDa (see Figure 3). Treatment of purified EBP with glycosidases did not change the relative migration of these bands suggesting that they did not arise simply by variation in N- or O-linked carbohydrate. C-terminal sequencing previously revealed the 22kDa band as a polypeptide of 150 amino acids designated EBP^{1-150}. The 24 kDa band was found to be 159 amino acids long as evidenced by C-terminal sequencing. This form was designated EBP^{1-159}.

CHO cells expressing EBP were studied for the presence of membrane-bound forms of EBP. The recombinant CHO cell line 36.44 was established by transfection of CHOd- cells with the plasmid pDSRα-EBP. Cells were grown in suspension media using DMEM:F12 media supplemented with 1X non-essential amino acids and 1X penicillin, streptomycin, glutamine with 10% heat inactivated dialyzed fetal bovine serum. Aliquots of 10^6 cells were treated with 4 µg/mL phospholipase C (Calbiochem, La Jolla, CA) in 1mL phosphate buffered saline at 37°C for various times. Cells were pelleted at 14,000 RPM for 1 minute. Supernatants were removed for analysis. Cell pellets were lysed in RIPA buffer (150 mM NaCl; 1% NP-40; 0.5% deoxycholate; 50 mM Tris-HCl pH 8.0; 0.1% SDS; 1.74 µg/mL PMSF; 1ng/mL each of aprotinin, pepstatin and leupeptin; 18.4 µg/mL orthovanadate). Samples were applied to a 14% Tris-Glycine gel, blotted to a PVDF membrane and probed with a rabbit anti-EBP polyclonal antibody. Figure 4 shows that EBP was released into the supernatant in the presence of phospholipase C. These experiments suggested that the 27 kDa form of EBP had a glycophospholipid anchor.

EXAMPLE 5

EBP Analogs

In addition to the different forms of recombinant EBP made from the full-length EBP gene, analogs of EBP were constructed having varying polypeptide lengths. In particular, EBPs having 167, 171 and 180 amino acids were constructed as follows. Oligonucleotides 421-12, 421-13 and 421-14 were synthesized for use as PCR primers to introduce termination codons following amino acid 180, 171 and 167, respectively. PCR reactions were done as described in Example 4B using each of those primers and the pDSRα-EBP plasmid and oligonucleotide 386-2.

421-12 5'-GAATTCTCTAGATTATCATGGAAGGAGCAGCACAGTCCAG-3'
(SEQ. ID. NO. 14)

421-13 5'-GAATTCTCTAGATTATCATGGGAAGAGGCGTGGGGCAGC-3'
(SEQ. ID. NO. 15)

421-14 5'-GAATTCTCTAGATTATCATGGGGCAGCACTGTGACCGATGC-3'
(SEQ. ID. NO. 16)

The resulting analogs were expressed in CHO cells transfected with the altered DNA sequences using procedures described for the expression of EBP. Cells were grown to confluence in the presence of serum whereupon the media was switched to serum-free and allowed to accumulate. At 48 hours the conditioned medium was collected and the adherent cells were lysed. Aliquots of the conditioned media and lysates were fractionated by polyacrylamide gel electrophoresis and subjected to Western blotting. EBP^{1-187} and EBP^{1-180} displayed a similar distribution of protein reacting with the antibody in lysates and supernatants. Cells expressing EBP^{1-171} and EBP^{1-167} had accumulation of EBP in

the supernatants, but not in the lysates. EBP analogs are analyzed for binding to *eck*-x by BIAcore as describe in Example 2 and for phosphorylation activity of *eck* receptor as described in Example 6.

EXAMPLE 6

Interactions of recombinant EBP with the *eck* receptor

A. Crosslinking studies.

CHO-cell derived EBP was radiolabelled with ^{125}I as described below for use in crosslinking and binding studies. Five or 10 µg of EBP in 0.1M sodium phosphate ($NaPO_4$, pH 8.0) was added to 5 mCi of dried ^{125}I-Bolton-Hunter reagent (NEN, Boston, MA) in a final volume of 50 µl or 100 µl, and the tube was incubated at 4°C for 1 h. The reaction was terminated by addition of 0.3 ml of 0.2M glycine in 0.1M $NaPO_4$, followed by incubation at 4°C for 5 min. Labeled protein was separated from unincorporated reagent by gel filtration chromatography on a 10 ml PD10 column containing Sephadex G-25 M (Pharmacia) equilibrated with 0.1M $NaPO_4$- 0.25% gelatin. Specific activity of the ^{125}I-EBP ranged from 4 to 19 cpm/pg.

Crosslinking of EBP to either LIM 2405 (a cell line naturally expressing the *eck* receptor) or CHO 19.32 (Chinese hamster ovary cells transfected with a clone of the full length *eck* receptor) was carried out as follows. CHO cells were grown in suspension to a density of approximately $5x10^5$ cells/ml in media. LIM2405 cells were grown in RPMI 1640 media containing 5% FCS, 1 µg/ml insulin, 1 µg/ml hydrocortisone, 10 µM thioglycerol and 2 mM L-glutamine to approximately 90% confluency in T-175 flasks and removed by scraping for use in the crosslinking studies. Cells were spun down and resuspended in PBS to give a single cell suspension. For each crosslinking reaction, $2x10^6$ cells were mixed

with approximately 20 ng of ^{125}I-EBP in a total volume of 1 ml. This mixture was incubated at 4°C for 1 h to allow binding of EBP to cell surface receptors before the addition of 20 µl of 10mM disuccinimidyl suberate (DSS) as a crosslinking agent. Cells were then washed three times in binding buffer, collected by centrifugation, and the amount of radioactivity incorporated into the cell pellets was counted to assess the degree of crosslinking. The cells were then lysed by treatment with 100 ul PBS, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO) for 10 min at 4° C. The insoluble material was removed by centrifugation and the soluble fraction containing the receptor/ligand complex was collected. These samples were either run directly on SDS-PAGE or first immunoprecipitated with an antibody directed against the C-terminal portion of the *eck* receptor (Lindberg et al. supra). Competition with either unlabeled EBP or an irrelevant protein (FGF) was carried out by mixing the competitor with the labeled EBP prior to addition to the cells.

As shown in Figure 5, ^{125}I-rEBP could be crosslinked to CHO 19.32 cells (lane 1) but not to the untransfected cells (lane 2). Crosslinking resulted in the detection of a 145 kDa protein, as well as higher molecular weight forms which may represent receptor dimers or higher order complexes. Unlabelled rEBP at 50-fold molar excess was able to compete for binding and crosslinking (lane 3). In separate experiments, recombinant fibroblast growth factor at concentrations of 1 µg/mL had no effect on rEBP crosslinking. Similar crosslinking results were obtained for the LIM 2405 cell line.

B. Binding studies.

To measure association kinetics, CHO 19.32 cells (2×10^6 cells/ml) were incubated with 3.8 nM ^{125}I-EBP in PBS-BSA (1mg/ml), at 0°C. Aliquots were removed, and cell-bound ^{125}I-EBP was determined by centrifugation through sucrose gradients. Nonspecific binding was measured from parallel reactions containing 380 nM unlabelled EBP.

Equilibrium binding constants were determined by incubating CHO 19.32 or LIM 2405 cells (0.5×10^6 cells/ml) with varying amounts of ^{125}I-EBP at 0°C for 1 h, bound EBP was determined as above, and the data was analyzed by the method of Scatchard (*Annal. N.Y. Acad. Sci.* 51, 660 (1949)). Nonspecific binding was determined from parallel reactions containing a 50 fold excess of unlabeled EBP.

A Scatchard analysis of the steady-state binding of ^{125}I-rEBP to LIM2405 cells revealed that there was apparently a single class of receptors on the cell surface with a K_d of 2.8×10^{-8} M $\pm$ 0.3×10^{-8}. On average, the LIM2405 cells contained 1.3×10^6 EBP receptors at the cell surface. BIAcore analysis of EBP binding to immobilized *eck*-x surfaces resulted in an estimated K_d of 2.4×10^{-8} M $\pm$ 0.4×10^{-8}.

C. p130^{eck} autophosphorylation studies.

The LIM 2405 colorectal carcinoma cell line (Whitehead et al. *Immunol. and Cell Biol.* 70, 227 (1992)) was maintained in RPMI 1640 containing 5% FBS, 1 µg/ml insulin, 10 µg/ml hydrocortisone and 10 µM α-thioglycerol, and subpassaged by trypsination and dilution (1:5) into fresh media. Prior to assay 2.5×10^5 LIM 2405 cells were seeded into 6-well dishes (Falcon # 3046) and incubated for 24 hr at 37°C. The media was discarded and replaced with serum-free RPMI 1640 containing 0.03% BSA, 1 µg/ml insulin, 10 µg/ml

hydrocortisone and 10 μM α-thioglycerol, then incubated for 12-18 hr. In some experiments the cell cultures were labelled with ^{32}P-orthophosphate (2 mCi/ml) in phosphate-free RPMI 1640 (Flow) for 2-3 hr prior to treatment. Growth factor stock solutions were prediluted at varying concentrations into 2.0 ml of serum-free media, then warmed to 37°C. Cell cultures were removed from the incubator, and the supernatant media discarded. Treatments were promptly added and the cell cultures incubated at 37°C for 10-15 min. The cultures were then removed from the incubator, placed on ice, and the culture supernatant aspirated.

The treated cell cultures were chilled to 0°C then washed once with ice-cold PBS (GIBCO). The residual PBS was aspirated and the cells were lysed by the addition of 1.0 ml of ice-cold RIPA buffer (10 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 0.1 % sodium dodecyl sulfate, 1% NP-40, 1% deoxycholate, 1% Trasylol, 2 mM EDTA, 50 mM sodium fluoride and 100 mM sodium orthovanadate). After a 10 min incubation the lysates were transferred to 1.5 ml tubes and clarified by centrifugation for 30 min at 10,000 X *g*. The clarified lysate supernatants were transferred to new tubes and immunoprecipitated with 1.0 μg/ml of affinity purified rabbit anti-Eck C-terminal domain antibody for 2 hr at 0°C. Immune complexes were adsorbed to Protein-G Sepharose beads (Pharmacia) at 4 °C for 30 min, washed twice with ice-cold RIPA buffer, once with 0.1 M Tris-HCl, pH 8.0, containing 0.5 M LiCl, and once with RIPA. The resulting immunoprecipitates were solubilized with SDS-PAGE sample buffer and stored for further analysis.

The anti-*eck* immunoprecipitates from treated and untreated LIM 2405 cell lysates were resolved on 7.5% polyacrylamide gels as previously described (Boyle et al. *Meth. Enzymol.* 201, 110 (1991)). After electrophoresis, the gels were electroblotted (Kamps

Meth. Enzymol. 201, 110 (1991)) onto Immobilon P (Millipore) and the blots were incubated for 1 hr in Tris-buffered saline containing 0.1 % Tween-20 (TBST) and 5% BSA to block non-specific binding sites on the membrane. Primary antibodies, either anti-phosphotyrosine antibody (4G10, UBI, Lake Placid, NY), or anti-*eck* C-terminal, were diluted to 1.0 µg/ml in TBST containing 3% BSA, 1% ovalbumin and incubated with the blots for 1 hr at room temperature. After this, the blots were rinsed with TBST, then washed once for 10-15 min, then twice for 5 min, each with TBST. The blots were then incubated with a 1:5000 dilution of secondary antibody coupled to horseradish peroxidase (Amersham, Arlington Heights, IL) in TBST alone for 20-30 min, then washed as before using TBST. Immune complexes were detected by chemiluminescent exposure (ECL, Amersham) to Kodak X-OMAT X-ray film at room temperature for 0.5-5 min.

Eck receptor immunoprecipitates from ^{32}P-labelled LIM 2405 cells were resolved by SDS-PAGE, and the gel dried directly without fixation. After exposure to X-ray film the labelled *eck* receptor band was isolated and phosphoamino acid content determined as described (Boyle et al. *Meth. Enzymol.* 201, 110 (1991))

CHO cell-derived rEBP stimulated *eck* receptor phosphorylation in intact LIM 2405 cells in a dose-dependent manner, with an optimal concentration between 100 ng/ml and 1 mg/ml (Figure 6, upper panel). There also appeared to be a modest dose-dependent decrease in the total cellular *eck* protein levels (Figure 6, lower panel), suggesting down regulation of the receptor after exposure to soluble EBP. Treatment of LIM 2405 cells with EBP does not result in spurious phosphorylation of the EGF receptor, nor does EGF treatment induce *eck* phosphorylation. Furthermore, when total cellular protein from LIM 2405 cells treated with rEBP was

analyzed, the only induced phosphoprotein is a Mr 130 kd polypeptide that corresponds to the mature *eck* receptor.

rEBP$^{1-150}$ from *E. coli* was also assayed for autophosphorylation of the *eck* receptor on LIM 2405 cells following the procedure used for CHO-cell derived rEBP. Upon treatment of cells with the same quantities of CHO-derived rEBP and *E. coli* derived EBP$^{1-150}$, it was observed that both forms of recombinant EBP were active in inducing phosphorylation.

EXAMPLE 7

Formulation of Recombinant EBP

Measuring the ability of EBP to bind to the immobilized soluble extracellular domain of *eck* using BIAcore, it was determined that dilute solutions of purified EBP rapidly lost the ability to bind to the *eck* receptor unless formulated with a protective agent such as a detergent. Recombinant CHO EBP diluted into PBS to 100 µg/ml and incubated for 2 hours at 3°C lost approximately 50% of its *eck* binding activity. This loss of activity can be avoided by formulating the EBP in a detergent such as 1mM CHAPS, 0.1% NP-40, 0.1% tween 20, 0.1% triton-X 100, or 0.1% tween 80. (see figure 7). The loss of *eck* binding activity in diluted EBP can also be avoided by incubating the protein with other protein carriers such as fetal calf serum.

The *eck* binding activity of at EBP 2-5mg/ml kept in detergent solutions is stable for at least one week at 3°C, but protein solutions in the 10-500µg/ml range will lose activity if stored at -20°C, or if they are subjected to multiple freeze thaw cycles.

EXAMPLE 8

Expression of EBP and *eck* receptor in tissues and cell lines

Expression of EBP has been studied at the mRNA level in various rat tissues and organs using procedures described in Lindberg et al., supra. EBP is expressed most highly in the lung, intestine, liver, ovary and kidney. Expression was also detected at lower levels in muscle, stomach, and brain.

Expression studies have been done for both EBP and the *eck* receptor in cell lines. EBP expression was tested for by immunoblotting cell supernatants in a Western analysis with affinity purified anti-human EBP monoclonal antibodies. As shown in Table 2, EBP is found in many carcinoma cells. *eck* expression was tested for by several methods, including immunoblotting of whole cell lysates and immunoprecipitation with anti-*eck* antibody from cell lysates. (Lindberg et al., supra) The results in Table 2 show that *eck* is expressed in many cell lines of epithelial origin, and in addition, is found in fibroblasts and melanoma cell lines.

TABLE 2

Cell lines that express EBP:

Cell line	Cell type
CaCo2	Colon adenocarcinoma
FADU	Squamous carcinoma
T47D	Breast carcinoma
MDAMD361	Breast adenocarcinoma
THP-1	Monocytic leukemia
SKBR3	Breast adenocarcinoma
CaOV4	adenocarcinoma
MDAMB453	Breast adenocarcinoma
Caki1	Kidney carcinoma
HBL100	Breast
HT29	Colon adenocarcinoma
JEG1	Choriocarcinoma
293	Embryonic kidney
A704	Kidney adenocarcinoma
Caki2	Kidney adenocarcinoma
CaOV3	Ovarian adenocarcinoma
SKOV3	Ovarian adenocarcinoma
A172	Glioblastoma
A431	Epidermal carcinoma
BSC1	Kidney
BT20	Breast carcinoma
PC3	Prostate adenocarcinoma
JAR	Choriocarcinoma
A498	Kidney carcinoma
LNCaP	Prostate adenocarcinoma
BT474	Breast carcinoma
SW480	Colon adenocarcinoma
SW620	Colon adenocarcinoma
MCF7	Breast adenocarcinoma
T24	Bladder carcinoma
5637	Bladder carcinoma
Du145	Prostate carcinoma
SKNSH	Neuroblastoma
L929	Connective tissue
G401	Kidney Tumor

TABLE 2 (Con't)

Cell lines that express *eck*:

Cell line	Cell type
THP-1	Monocytic leukemia
CCD11Lu	Lung
HT29	Colon adenocarcinoma
SKOV3	Ovarianadenocarcinoma
A172	Glioblastoma
A431	Epidermal carcinoma
JAR	Choriocarcinoma
GM4312A	Fibroblast
Wi38	Lung
UT7	Premegakaryocyte
CHOK1	Ovary
HS249T	Melanoma
M14	Melanoma
NIH3T3	Fibroblast

EXAMPLE 9
Biological Activities of EBP

A. Activity of EBP in Rat Wound Chamber Assay

The effects of recombinant CHO-derived and E.coli derived EBP on granulation tissue formation in subcutaneously implanted wound chambers in rats was studied.

Male Sprague-Dawley specific pathogen free rats (300-350g; Charles River Breeding Laboratories, Inc.) were used for this study. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) by intraperitoneal injection. Using aseptic surgical technique, a 3 cm midline incision was made on the dorsal surface of the rat. A single pocket was formed under the panniculus carnosis by blunt dissection on one side of the animal. A 3 cm long x 1 cm diameter sterile stainless steel wire mesh cylinder was then inserted subcutaneously. The wound chambers used were similar to those described by Schilling et. al. (*Surgery* 46, 702-710 (1959)) and utilized by Hunt et. al. (*Am. J. Surg.* 114, 302-307 (1967)) as wound healing models. The incision was closed with wound clips. The rats survived the operation well with no evidence of postoperative discomfort. Beginning 3 days after wound chamber implantation, the rats were randomly divided into 5 groups. At this time daily injections of 1) 10 µg CHO-derived EBP, 2) 1 µg CHO-derived EBP, 3) 8 µg E.coli-derived EBP, 4) 5 µg recombinant platelet derived growth factor (PDGF), or 5) 0.1 ml sterile PBS and 1 mM CHAPS were begun and continued for a total of 9 days. The injections were made directly into the wound chamber through a silicone septum at the outer edge of the chamber. Twelve days after chamber implantation, the rats were sacrificed by CO_2 asphyxiation. Immediately after sacrifice, the chambers were removed surgically

and carefully opened. The enclosed granulation tissue was then weighed, and stored at -70°C for future processing.

Granulation tissue samples were thawed and homogenized with a Polytron homogenizer in 2 ml of ice-cold distilled water for approximately 1 minute. Next, 2 ml of ice-cold 10% trichloroacetic acid was added to the homogenate and incubated at 4°C for 1 hour. The TCA-precipitated samples were centrifuged (500g, 10 minutes) at 4°C, washed once with 2 ml of ice-cold 5% TCA, and finally washed with 2 ml of ice-cold 95% ethanol containing 50 mM sodium acetate. Samples were defatted twice with 2 ml of ethanol:ether (3:1, v/v) at 20°C. After each defatting procedure, samples were centrifuged (500xg, 10 minutes) and the supernate discarded. The pellet was then dissolved in 1N sodium hydroxide and brought up to a volume of 10 ml.

Total protein was determined by taking an aliquot of the solubilized granulation tissue and assaying the sample according to the method of Bradford (Anal. Biochem. 72, 248-251 (1976)) Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used for a protein standard curve.

Total DNA content was determined as deoxyribose by the colorimetric method of Burton (Biochem. J. 62, 315 (1956)). Briefly, a 1 ml aliquot of the solubilized sample (in N NaOH) was neutralized with the addition of 0.5 ml of 2 N HCl. A 1 ml aliquot of the neutralized solution was extracted in 10% perchloric acid at 90°C for 15 minutes. A 1 ml portion of the final extract was added to 2 ml activated diphenylamine (Sigma Chemical, St. Louis, MO) reagent and absorbance at 600 nm was measured after overnight incubation at room temperature. Hydrolyzed calf thymus DNA (Sigma) was used to construct the standard curve. The DNA content was measured as a rough index of the number of

cells in the wound chamber, with the realization that cell type and cell cycle status also influence DNA content.

Total glycosaminoglycans (GAGs) were determined using chondroitin sulfate as a standard. A spectrophotometric assay for GAGs was run utilizing a change in absorption spectrum of the dye 1,9- dimethyl methylene blue (Farndale et al. Conn. Tiss. Res. 9, 247-248 (1982)).

Total collagen content was determined as hydroxyproline (an index of collagen content) after hydrolysis of the solubilized granulation tissue in 6N HCl at 110°C for 24 hours. Aliquots of 200 µl sample hydrolyzate were dried down, reconstituted in 200 µl of buffer and analyzed for hydroxy-L-proline by amino acid analysis using a Beckman 6300 amino acid analyzer. Quantitation was done using an external standard of hydroxy-L-proline (Sigma).

The data was analyzed by one-way analysis of variance. Significant differences were then analyzed comparing individual group means using a two-tailed unpaired student's t test. Statistical significance was defined as $p<0.05$. All values are expressed as mean ± SEM.

CHO-derived rEBP at a dose of 10 µg per chamber per day for 9 consecutive days and rPDGF at a dose of 5 µg per day (x 9 days) both significantly increased the wet weight, total protein, total DNA and total GAG content of the chamber granulation tissue as compared to vehicle-treated (PBS and 1 mM CHAPS) control rats (see Table 3). All chambers were harvested 12 days after implantation. This timepoint is at the peak of granulation tissue formation and at the early stages of collagen formation. CHO-derived rEBP at a dose of 1 µg per chamber per day showed a significant increase in granulation tissue wet weight and GAG content over

vehicle treated control rat chambers. However, there were no significant differences against control in the total protein, DNA, and hydroxyproline content for the 1 µg rEBP dose.

The accumulation of collagen is probably the single most important factor contributing to wound strength. PDGF treated chambers showed an 82% increase in hydroxyproline content (and therefore collagen synthesis) over control chambers. CHO-derived rEBP showed a 21% increase in hydroxyproline at 1 µg/chamber/day, and a 35% increase in hydroxyproline at 10 µg/chamber/day, although these increases were not statistically significant.

TABLE 3

Effects of CHO-Derived EBP on Granulation Tissue Formation in Wound Chambers in Rats

TREATMENT	WET WEIGHT (mg)	TOTAL PROTEIN (mg)	DNA (µg)	HYDROXY-PROLINE (µg)	TOTAL GYLCOSAMINO-GLYCANS (µg)	CHANGE IN BODY WEIGHT (g)
Control	158.6 ± 17.9	2.76 ± 0.27	153 ± 20	263.5 ± 29.1	383.3 ± 41.5	+53.8 ± 5.4
PDGF 5ug	417.4 ± 81.8**	7.46 ± 1.52**	356 ± 64**	478.4 ± 85.9*	725.2 ± 104.4**	+45.4 ± 2.8
CHO-EBP 10µg	398.2 ± 70.9**	7.15 ± 1.23**	650 ± 163*	354.5 ± 63.7	627.9 ± 92.4*	+50.3 ± 5.2
CHO-EBP 1µg	238.0 ± 24.1*	3.88 ± 0.44	232 ± 41	318.7 ± 31.7	589.2 ± 68.4*	+48.5 ± 4.9

All Values are Mean ± SEM

*$p<0.05$ by two-tailed unpaired Student's t test vs. control group

**$p<0.01$ by two-tailed unpaired Student's t test vs. control group

B. Activity of EBP in Murine Hematopoiesis

Unfractionated bone marrow suspensions from BDF mice were plated into serum free culture medium (Iscove's modified Dulbecco's medium) in 0.3% agarose and incubated at 37°C, 5% CO_2 for ten days at a cell concentration of $1X10^5$/ml in 96 well plates. Total volume per cell was 50 µl. EBP and other growth factors were added in the amounts indicated and colonies were scored at the tenth day of incubation. The results are shown in Figure 8.

EBP at 1 µg/ml could potentiate murine IL-3 dependent murine CFU-C formation about 2-fold compared to IL-3 alone. CFU-C represents CFU-G, CFU-GM and CFU-M. EBP alone showed no stimulation of CFU-M or BFU-E/CFU-E.

C. Activity of EBP in Colon Crypt Assay

Colons from five (BDF) mice were removed and crypts isolated using a non enzymatic (EDTA) extraction method. Briefly, colons are washed and allowed to sit for 20 min. in a solution of PBS containing .04% sodium hypochlorite. Crypts are isolated by incubating the tissue for 1 hour at 37°C in a solution of PBS containing 3mM EDTA and 0.5 mM DTT. Crypts are then subjected to pancreatin digestion (0.2% in PBS) for 90 min. at 37°C in order to obtain a single cell suspensing. Cells are washed in PBS, counted and viability determined by trypan blue exclusion (85% in this experiment). Cells are then plated in a top layer of 0.37% Sigma low melt agar onto a bottom layer of 0.5% agar. The RPMI media used in the agar includes the presence of 1X ITS (insulin, transferrin, and selenium, Gibco). Incubations were done in the presence of 1% fetal calf serum (FCS) and the indicated growth factors at the following concentration: TGFα, 50ng/ml; bFGF, 60ng/ml; EBP, 500ng/ml. Control incubations lacked

growth factors and/or FCS. Each of the various culture conditions was performed in triplicate and scored every several days.

Positive results were obtained after crypt cells were incubated with 1% FCS and EBP or 1% FCS and bFGF. In both conditions, the clusters of cells were large and the individual cells within the clusters appear healthy. In some cases the clusters were crypt-like shaped. When both EBP and bFGF were added together with FCS, fewer and smaller clusters appeared compared to when either growth factor was added alone in the presence of 1% FCS. Initially, the combination of EBP and bFGF appeared to be inducing cell growth but this stimulation in growth was short lived. In the plates with EBP alone (no serum) there were occasional large clusters, however these were looser, the cells were larger and roughly half the cells were nonviable within the cluster.

These results suggest that EBP is involved in the proliferation, differentiation or reformation of colon crypt cells.

TABLE 4

Activity of EBP in Colon Crypt Assay

DAYS	TGFα	TGFα+EBP	EBP	bFGF	EBP+bFGF	1%FCS+EBP	1%FCS+bFGF	1%FCS+ EBP+bFGF	1%FCS	NT
2	--	--	--	--	+++	++	++1/2+	++	--	--
5	--	--	--	--	++	+++	++++	+++	+	--
7	--	--	+/--	--	+/--	++++	++	++	--	--
9	--	--	+/--	--	--	+++	++++	++	--	--
12	--	--	+/--	--	+/--	+++	++++	++	--	--

NT - not treated

D. Activity of EBP on Primary Cultures of Hepatocytes

Hepatocytes were isolated by the *in situ* two step collagenase perfusion technique described by Seglen (*Methods in Toxicol.*, Vol. 1A, 231-243 (1993). Briefly, the perfused livers were dispersed in ice cold Hank's buffer, filtered through a nylon mesh, and hepatocytes separated from nonparenchymal cells by repeated centrifugation at 50 X g. The hepatocytes were determined to be greater than 85% viable by trypan blue exclusion. The hepatocytes were cultured on plates coated with rat tail collagen and plated in Williams E containing 5% FCS, 10^{-7} M insulin, 10^{-8} M dexamethason, glutamine, penicillin and streptomycin.

Cells were allowed to attach for 3-4 hours in serum containing media and then transferred to serum-free media. The appropriate concentrations of EBP or other growth factors were added and the cells were incubated for 24 to 48 hours in the presence of ^{3}H-thymidine. After incubation, the extent of ^{3}H-thymidine incorporation into cells was determined. The growth factors tested were keratinocyte growth factor (KGF) at 10 ng/ml, acidic FGF (aFGF) at 20 ng/ml, and EBP at 20ng/ml. The results are shown in Figure 9. The amount of ^{3}H-thymidine incorporation stimulated by EBP is about the same as that induced by aFGF. The combination of KGF and EBP did not stimulate hepatocyte growth over the level observed by KGF alone. In this particular experiment, the serum free value was unusually high (about 3-fold).

* * *

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Bartley, Timothy Dudley
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Boyle, William James
Welcher, Andrew Avery
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(A) MEDIUM TYPE: Diskette, 3.5 in., DS, 2.0 Mb

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: Macintosh OS 7.0

(D) SOFTWARE: Microsoft Word Version 5.1a

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 205 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Glu Phe Leu Trp Ala Pro Leu Leu Gly Leu Cys Cys Ser
            -15                 -10                  -5

Leu Ala Ala Ala Asp Arg His Thr Val Phe Trp Asn Ser Ser
                 1               5                   10

Asn Pro Lys Phe Arg Asn Glu Asp Tyr Thr Ile His Val Gln
                15                  20

Leu Asn Asp Tyr Val Asp Ile Ile Cys Pro His Tyr Glu Asp
25                  30                  35

His Ser Val Ala Asp Ala Ala Met Glu Gln Tyr Ile Leu Tyr
    40                  45                  50

Leu Val Glu His Glu Glu Tyr Gln Leu Cys Gln Pro Gln Ser
        55                  60                  65

Lys Asp Gln Val Arg Trp Gln Cys Asn Arg Pro Ser Ala Lys
            70                  75                  80

His Gly Pro Glu Lys Leu Ser Glu Lys Phe Gln Arg Phe Thr
                85                  90

Pro Phe Thr Leu Gly Lys Glu Phe Lys Glu Gly His Ser Tyr
95                  100                 105

Tyr Tyr Ile Ser Lys Pro Ile His Gln His Glu Asp Arg Cys
    110                 115                 120

Leu Arg Leu Lys Val Thr Val Ser Gly Lys Ile Thr His Ser
        125                 130                 135

Pro Gln Ala His Val Asn Pro Gln Glu Lys Arg Leu Ala Ala
            140                 145                 150

Asp Asp Pro Glu Val Arg Val Leu His Ser Ile Gly His Ser
                155                 160

Ala Ala Pro Arg Leu Phe Pro Leu Ala Trp Thr Val Leu Leu
165                 170                 175

Leu Pro Leu Leu Leu Leu Gln Thr Pro
    180                 185
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGCATATGG ATCGCCACAC CGTCTTCTGG

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGGATCCT TATCACGGGG TTTGCAGCAG CAGAA 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAAGGATCCC TATTATGCTG CAAGTCTCTT CTCCTG 36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATTCAAGC TTCAGGCCCC GCGCTATGGA G 31

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCTCTA GATCATCACG GGGTTTGCAG CAGCA 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCTTAGATC TCC 13

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATTGGAGAT CTA 13

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATTCCAGAC GCTGTCCCCG GAGGGATCCG GCAACTGAG

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGACTCAGT TGCCGGATCC CTCCGGGGAC AGCGTCTGG 39

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1480 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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GCGGAGAAAG CCAGTGGGAA CCCAGACCCA TAGGAGACCC GCGTCCCCGC TCGGCCTGGC      60

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               Met Glu Phe Leu Trp Ala Pro Leu Leu Gly Leu Cys
                1               5                   10

TGC AGT CTG GCC GCT GCT GAT CGC CAC ACC GTC TTC TGG AAC AGT TCA       157
Cys Ser Leu Ala Ala Ala Asp Arg His Thr Val Phe Trp Asn Ser Ser
        15                  20                  25

AAT CCC AAG TTC CGG AAT GAG GAC TAC ACC ATA CAT GTG CAG CTG AAT       205
Asn Pro Lys Phe Arg Asn Glu Asp Tyr Thr Ile His Val Gln Leu Asn
    30                  35                  40

GAC TAC GTG GAC ATC ATC TGT CCG CAC TAT GAA GAT CAC TCT GTG GCA       253
Asp Tyr Val Asp Ile Ile Cys Pro His Tyr Glu Asp His Ser Val Ala
45                  50                  55                  60

GAC GCT GCC ATG GAG CAG TAC ATA CTG TAC CTG GTG GAG CAT GAG GAG       301
Asp Ala Ala Met Glu Gln Tyr Ile Leu Tyr Leu Val Glu His Glu Glu
                65                  70                  75

TAC CAG CTG TGC CAG CCC CAG TCC AAG GAC CAA GTC CGC TGG CAG TGC       349
Tyr Gln Leu Cys Gln Pro Gln Ser Lys Asp Gln Val Arg Trp Gln Cys
            80                  85                  90

AAC CGG CCC AGT GCC AAG CAT GGC CCG GAG AAG CTG TCT GAG AAG TTC       397
Asn Arg Pro Ser Ala Lys His Gly Pro Glu Lys Leu Ser Glu Lys Phe
        95                  100                 105

CAG CGC TTC ACA CCT TTC ACC CTG GGC AAG GAG TTC AAA GAA GGA CAC       445
Gln Arg Phe Thr Pro Phe Thr Leu Gly Lys Glu Phe Lys Glu Gly His
    110                 115                 120

AGC TAC TAC TAC ATC TCC AAA CCC ATC CAC CAG CAT GAA GAC CGC TGC       493
Ser Tyr Tyr Tyr Ile Ser Lys Pro Ile His Gln His Glu Asp Arg Cys
125                 130                 135                 140

TTG AGG TTG AAG GTG ACT GTC AGT GGC AAA ATC ACT CAC AGT CCT CAG       541
Leu Arg Leu Lys Val Thr Val Ser Gly Lys Ile Thr His Ser Pro Gln
                145                 150                 155
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GCC CAT GTC AAT CCA CAG GAG AAG AGA CTT GCA GCA GAT GAC CCA GAG 589
Ala His Val Asn Pro Gln Glu Lys Arg Leu Ala Ala Asp Asp Pro Glu
160 165 170

GTG CGG GTT CTA CAT AGC ATC GGT CAC AGT GCT GCC CCA CGC CTC TTC 637
Val Arg Val Leu His Ser Ile Gly His Ser Ala Ala Pro Arg Leu Phe
175 180 185

CCA CTT GCC TGG ACT GTG CTG CTC CTT CCA CTT CTG CTG CTG CAA ACC 685
Pro Leu Ala Trp Thr Val Leu Leu Leu Pro Leu Leu Leu Leu Gln Thr
190 195 200

CCG TGAAGGTGTA TGCCACACCT GGCCTTAAAG AGGGACAGGC TGAAGAGAGG 738
Pro
205

GACAGGCACT CCAAACCTGT CTTGGGGCCA CTTTCAGAGC CCCCAGCCCT GGGAACCACT 798

CCCACCACAG GCATAAGCTA TCACCTAGCA GCCTCAAAAC GGGTCAGTAT TAAGGTTTTC 858

AACCGGAAGG AGGCCAACCA GCCCGACAGT GCCATCCCCA CCTTCACCTC GGAGGGACGG 918

AGAAAGAAGT GGAGACAGTC CTTTCCCACC ATTCCTGCCT TTAAGCCAAA GAAACAAGCT 978

GTGCAGGCAT GGTCCCTTAA GGCACAGTGG GAGCTGAGCT GGAAGGGGCC ACGTGGATGG 1038

GCAAAGCTTG TCAAAGATGC CCCCTCCAGG AGAGAGCCAG GATGCCCAGA TGAACTGACT 1098

GAAGGAAAAG CAAGAAACAG TTTCTTGCTT GGAAGCCAGG TACAGGAGAG GCAGCATGCT 1158

TGGGCTGACC CAGCATCTCC CAGCAAGACC TCATCTGTGG AGCTGCCACA GAGAAGTTTG 1218

TAGCCAGGTA CTGCATTCTC TCCCATCCTG GGGCAGCACT CCCCAGAGCT GTGCCAGCAG 1278

GGGGGCTGTG CCAACCTGTT CTTAGAGTGT AGCTGTAAGG GCAGTGCCCA TGTGTACATT 1338

CTGCCTAGAG TGTAGCCTAA AGGGCAGGGC CCACGTGTAT AGTATCTGTA TATAAGTTGC 1398

TGTGTGTCTG TCCTGATTTC TACAACTGGA GTTTTTTTAT ACAATGTTCT TTGTCTCAAA 1458

ATAAAGCAAT GTGTTTTTTC GG 1480

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Lys Arg Leu Ala Ala
 1               5
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Asp Arg His Thr Val Phe Asp Asn Ser Ser Asn Pro Lys Phe Arg Asn Glu Asp Tyr
 1               5                   10                  15

Ile His Val Gln
 20
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
GAATTCTCTA GATTTCATGG AAGGAGCAGC ACAGTCCAG                            40
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAATTCTCTA GATTATCATG GGAAGAGGCG TGGGGCAGC 39

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single-stranded

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAATTCTCTA GATTATCATG GGCAGCACT GTGACCGATG C 41

WHAT IS CLAIMED IS:

1. A purified and isolated polypeptide specifically binding the *eck* receptor and having substantially the same amino acid sequence as shown in SEQ. ID. NO. 1.

2. The polypeptide of Claim 1 which induces phosphorylation of the *eck* receptor.

3. The polypeptide of Claim 1 having an amino acid sequence as shown in SEQ. ID. NO. 1 terminating at position 150 or having substantially the same amino acid sequence as shown in SEQ. ID. NO. 1 terminating at position 150.

4. The polypeptide of Claim 3 wherein the amino acid sequence is from position +1 to 150 as shown in SEQ. ID. NO. 1.

5. The polypeptide according to Claim 1 having a methionine residue at position -1.

6. The polypeptide according to Claim 5 selected from the group consisting of [Met^{-1}] EBP$^{1-150}$ and [Met^{-1}] EBP$^{1-159}$.

7. The polypeptide according to Claim 1 selected from the group consisting of EBP$^{1-167}$, EBP$^{1-171}$ and EBP$^{1-180}$.

8. The polypeptide of Claim 1 which is characterized by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

9. The polypeptide of Claim 8 which is the product of CHO cell expression.

10. The polypeptide of Claim 8 wherein the exogenous sequence is a cDNA sequence.

11. The polypeptide of Claim 8 wherein the exogenous sequence is a genomic DNA sequence.

12. The polypeptide of Claim 8 wherein the exogenous sequence is a synthetic DNA sequence.

13. The polypeptide of Claim 8 wherein the exogenous DNA sequences is carried on an autonomously replicating DNA plasmid or viral vector.

14. The polypeptide of Claim 8 which is the product of *E. coli* expression.

15. The polypeptide of Claim 8 having an N-terminal methionine residue.

16. A DNA sequence encoding a polypeptide specifically binding the *eck* receptor, wherein said polypeptide has a methionine residue at position -1 and has substantially the same amino acid sequence as shown in SEQ. ID. NO. 1.

17. A DNA sequence according to Claim 16 encoding [Met^{-1}] EBP^{1-150} and [Met^{-1}] EBP^{1-159}.

18. A DNA sequence encoding EBP^{1-167}, EBP^{1-171} and EBP^{1-180}.

19. An isolated *eck* receptor-ligand complex.

20. The receptor-ligand complex according to Claim 13 wherein the ligand is the polypeptide according to Claim 1.

21. A method for detecting in crude samples a ligand capable of binding to a receptor comprising the steps of:

a) immobilizing a purified ligand binding domain of the receptor;

b) contacting the immobilized receptor with conditioned medium containing the ligand; and

c) monitoring the binding of the ligand to the immobilized receptor by a surface plasmon resonance detection system.

22. The method of Claim 21 wherein the receptor is the *eck* receptor.

23. A method of modulating the endogenous activity of an *eck* receptor in a mammal comprising administering to the mammal an effective amount of a ligand to the *eck* receptor to modulate the activity of said receptor.

24. The method according to Claim 23 wherein the ligand is the polypeptide of Claim 1.

25. The method according to Claim 23 wherein the modulation of said *eck* receptor activity regulates cellular functions comprising differentiation, proliferation and metabolism.

26. A method for identifying compounds that modulate the activity of an *eck* receptor comprising the steps of:

a) exposing cells exhibiting the receptor to known ligands for a time sufficient to allow formation of receptor-ligand complexes and induce signal transduction;

b) determining the extent of activity within the cells; and

c) comparing the measured activity to the activity in cells not exposed to the ligand.

27. A method for the treatment of cancer in a patient comprising administering a therapeutically effective amount of an *eck* receptor ligand.

28. A method for the treatment of cancer in a patient comprising administering a therapeutically effective amount of the ligand of Claim 27 wherein said ligand binds to, but does not phosphorylate the *eck* receptor.

29. A method for the treatment of cancer in a patient comprising administering a therapeutically effective amount of an *eck* soluble receptor.

30. A method as in either of Claims 27, 28 or 29 further comprising a therapeutically effective amount of chemotherapy or radiation therapy.

31. A method for the treatment of inflammation in a patient comprising administering a therapeutically effective amount of an *eck* soluble receptor.

32. A method for the treatment of inflammation in a patient comprising administering a therapeutically effective amount of an *eck* receptor

ligand wherein said ligand binds to, but does not phosphorylate the *eck* receptor.

33. A method for the treatment of a wound in a mammal comprising administering a therapeutically effective amount of an *eck* receptor ligand.

34. A method for increasing hematopoiesis in a mammal comprising administering a therapeutically effective amount of an *eck* receptor ligand.

35. The method according to Claim 34 wherein the ligand is the polypeptide of Claim 1.

36. A method for stimulating proliferation of colon cells comprising administering a therapeutically effective amount of an *eck* receptor ligand.

37. The method according to Claim 36 which is used in conjunction with cancer therapy.

38. The method according to Claim 36 wherein the ligand is the polypeptide of Claim 1.

39. A method for stimulating proliferation of hepatocytes comprising administering a therapeutically effective amount of an *eck* receptor ligand.

40. A method according to Claim 39 wherein the ligand is the polypeptide of Claim 1.

41. A pharmaceutical composition comprising a therapeutically effective amount of an *eck* receptor ligand and a pharmaceutically acceptable diluent, adjuvant or carrier.

42. A pharmaceutical composition comprising a therapeutically effective amount of an *eck* soluble receptor and a pharmaceutically acceptable diluent, adjuvant or carrier.

43. A composition comprising the polypeptide of Claim 1 and a detergent.

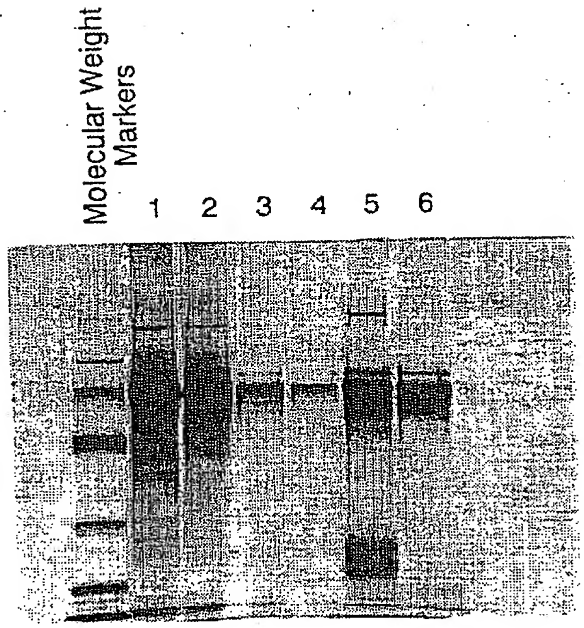

Molecular Weight Markers
1 2 3 4 5 6

FIG. 1A

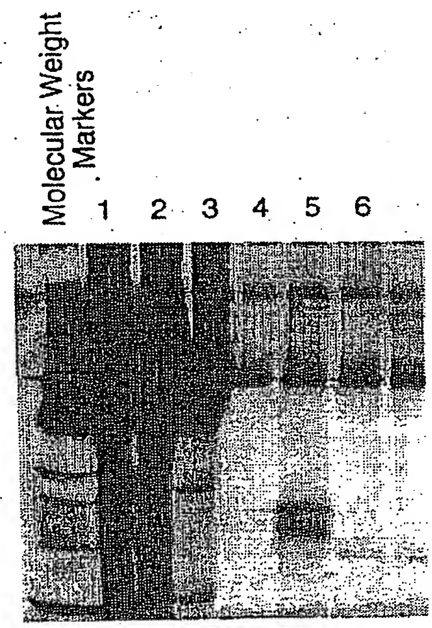

Molecular Weight Markers
1 2 3 4 5 6

FIG. 1B

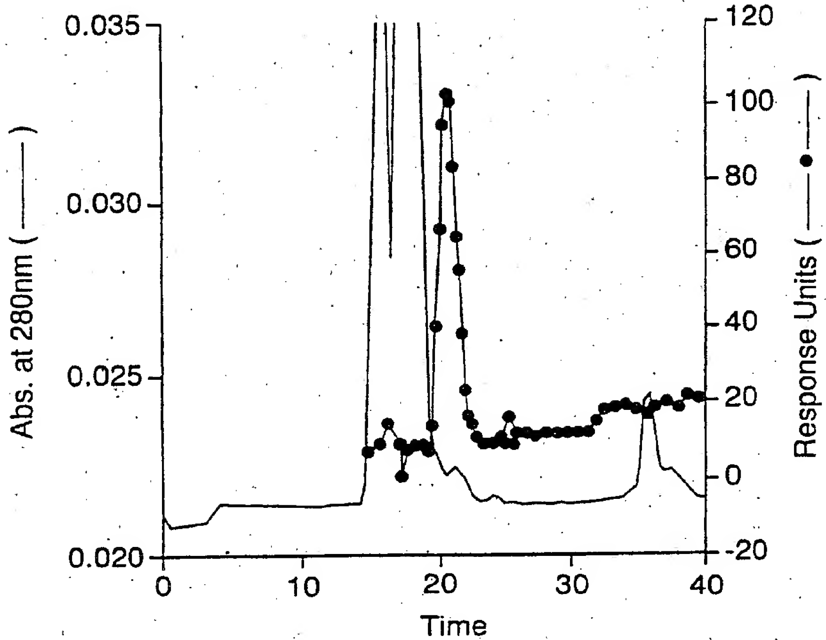

0.035
0.030
0.025
0.020
Abs. at 280nm (——)
120
100
80
60
40
20
0
-20
Response Units (—●—)
0
10
20
30
40
Time

FIG. 2

FIG. 3

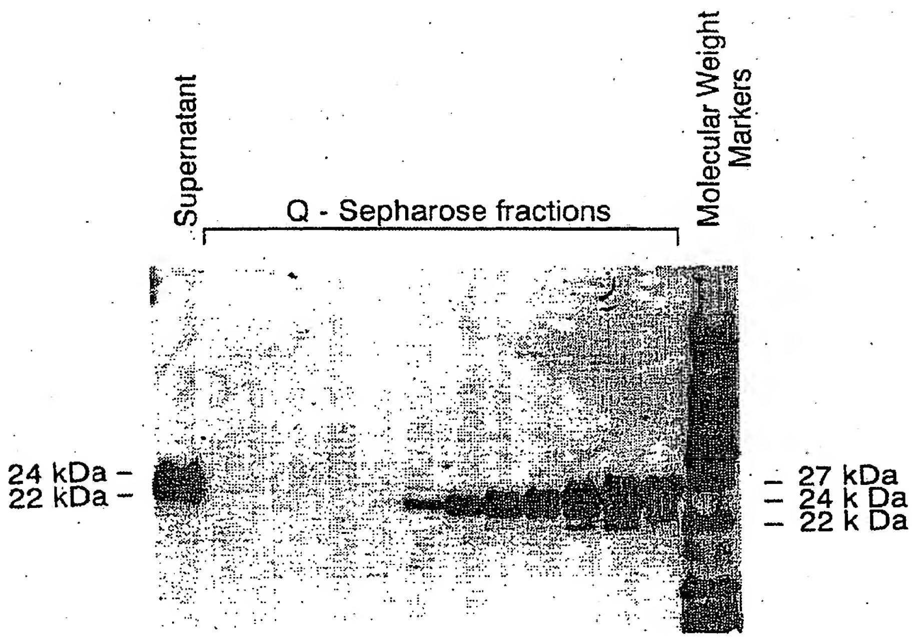

Supernatant
Q - Sepharose fractions
Molecular Weight Markers
24 kDa
22 kDa
27 kDa
24 k Da
22 k Da

FIG. 4

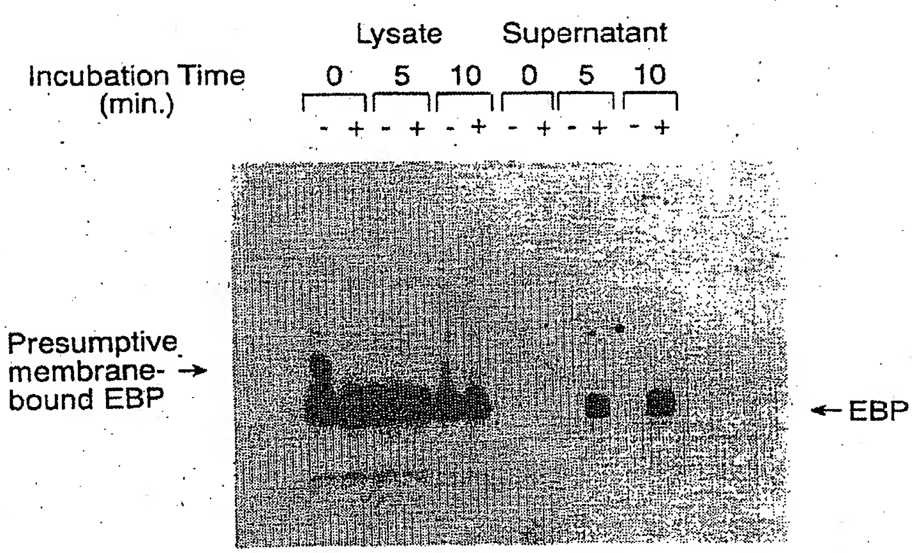

Lysate
Supernatant
Incubation Time (min.)
0 5 10 0 5 10
- + - + - + - + - + - +
Presumptive membrane-bound EBP
EBP

FIG. 5

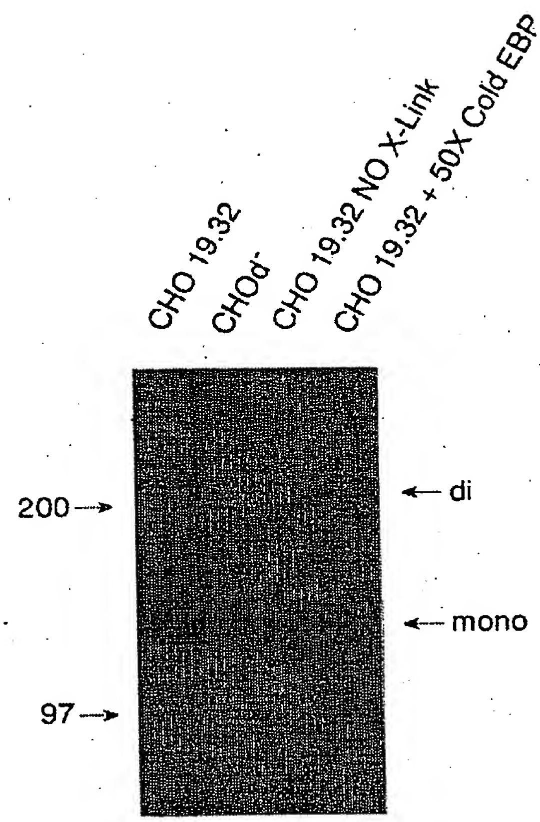
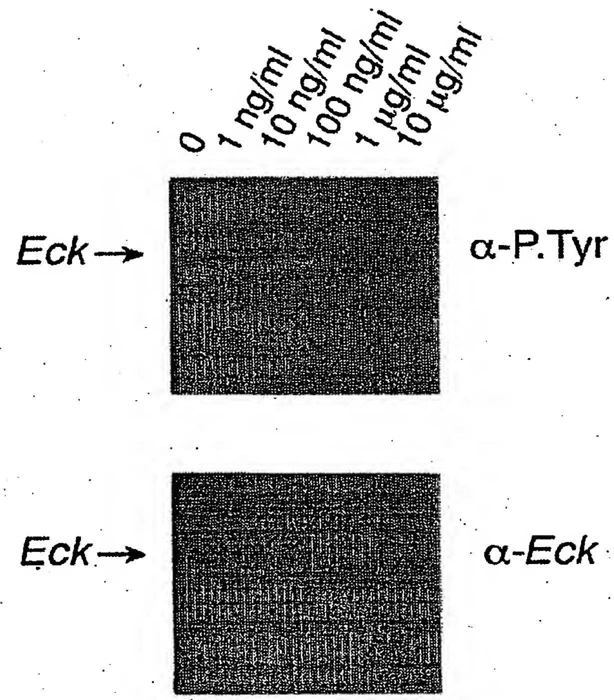

CHO 19.32
CHOd-
CHO 19.32 NO X-Link
CHO 19.32 + 50X Cold EBP
200
97
di
mono

FIG. 6



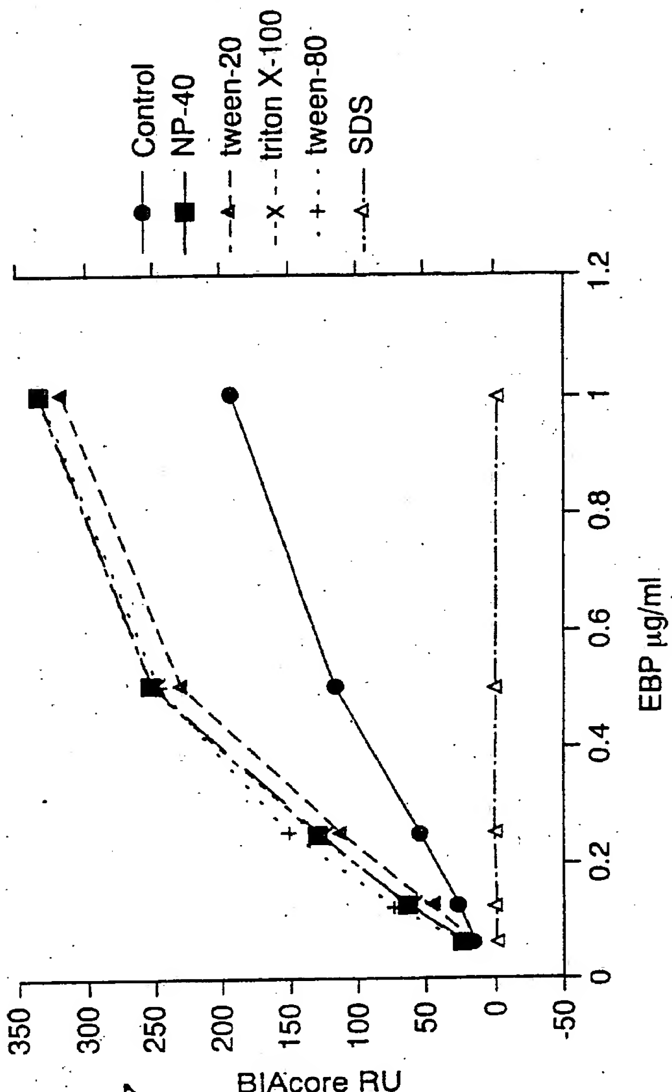

Control
NP-40
tween-20
triton X-100
tween-80
SDS
BIAcore RU
350
300
250
200
150
100
50
0
-50
0
0.2
0.4
0.6
0.8
1
1.2
EBP µg/ml

FIG. 7

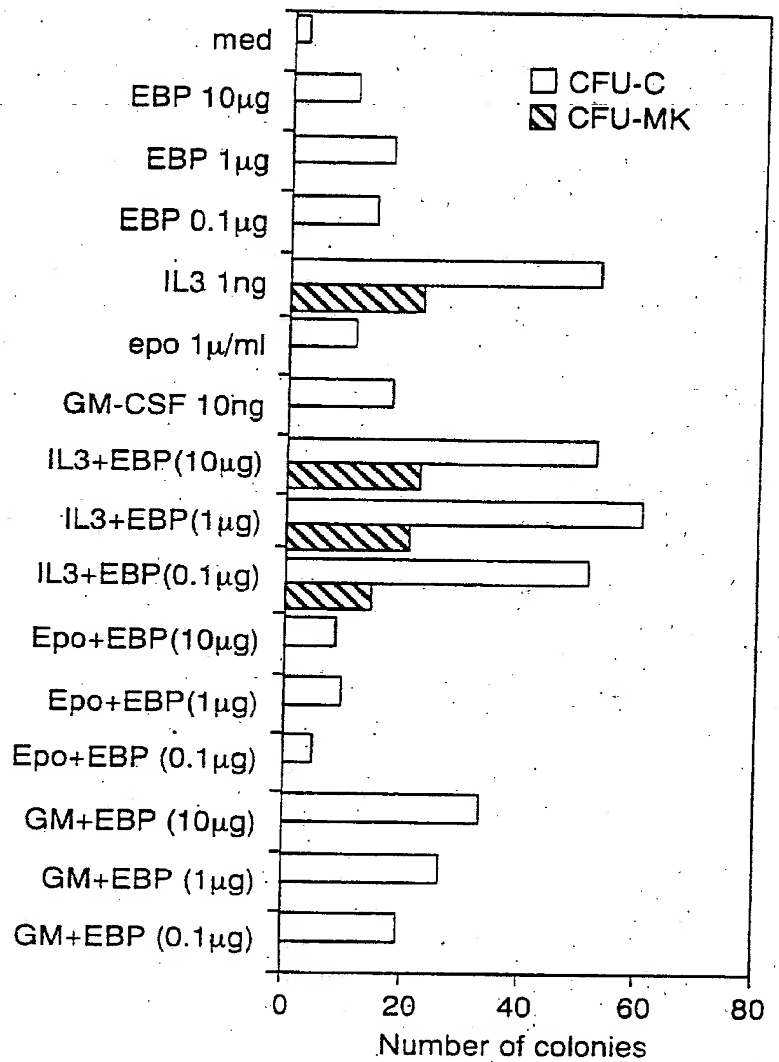

med
EBP 10µg
EBP 1µg
EBP 0.1µg
IL3 1ng
epo 1µ/ml
GM-CSF 10ng
IL3+EBP(10µg)
IL3+EBP(1µg)
IL3+EBP(0.1µg)
Epo+EBP(10µg)
Epo+EBP(1µg)
Epo+EBP (0.1µg)
GM+EBP (10µg)
GM+EBP (1µg)
GM+EBP (0.1µg)
CFU-C
CFU-MK
0
20
40
60
80
Number of colonies

FIG. 8

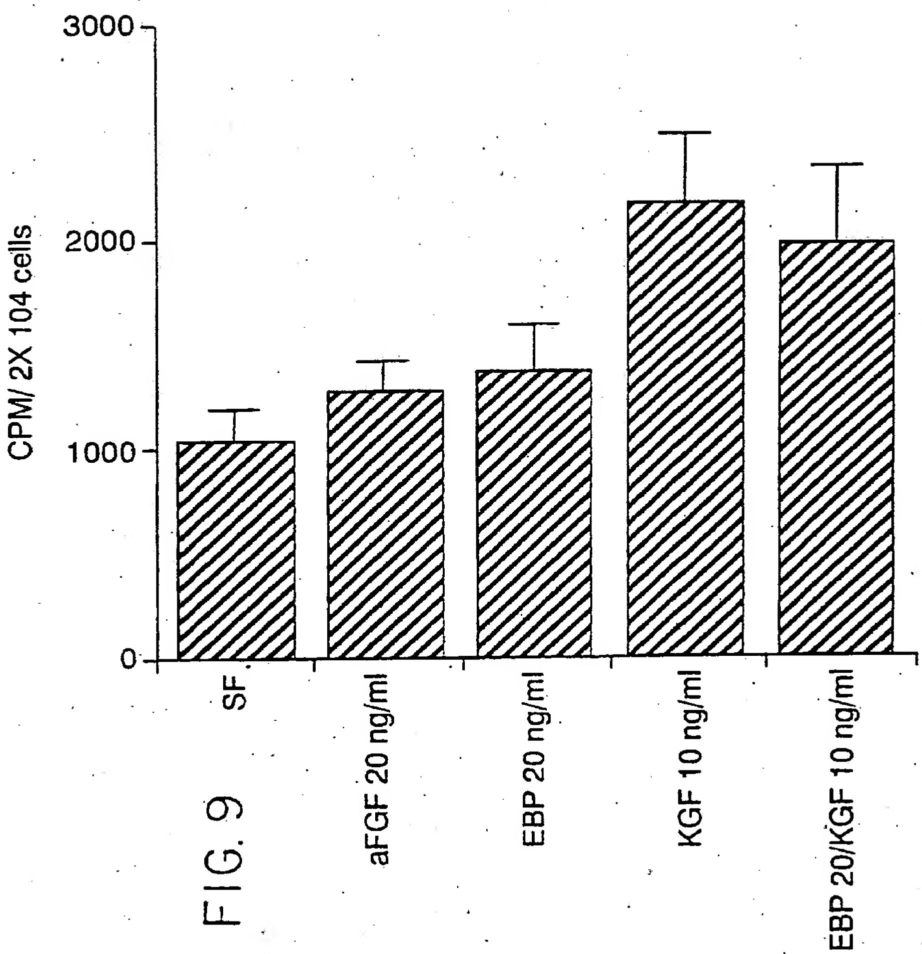

3000
2000
1000
0
CPM/ 2X 104 cells
SF
aFGF 20 ng/ml
EBP 20 ng/ml
KGF 10 ng/ml
EBP 20/KGF 10 ng/ml

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10879

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 37/52; C07K 13/00; C12N 15/12; C12Q 1/48
US CL :424/94.5; 435/7.1, 7.2; 514/12; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.5; 435/7.1, 7.2; 514/12; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog
search terms: eck, B61, ligand, bind?, receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Molecular and Cellular Biology, Vol. 10, No. 11, issued November 1990, L. B. Holzman et al., "A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein", pages 5830-5838, see page 5834.	1-18, 41, 43 — 19-26
X — Y	WO, A, 92/07094 (Dixit) 30 April 1992, see pages 11-12 and Figure 3A.	1-18, 41, 43 — 19-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1993	03 FEB 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer ERIC GRIMES D. Kyza for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Molecular and Cellular Biology, Vol. 10, No. 12, issued December 1990, R. A. Lindberg et al., "cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases", pages 6316-6324, see the entire document.	42 --- 19-26
Y	BioTechniques, Vol. 11, No. 5, issued 1991, U. Jonsson et al., "Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology", pages 620-627, see the entire document.	21, 22